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14. ABSTRACT Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. We have completed the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping. We have set up a structure for reviewing and approving biomarker proposals based on sound scientific principles and strong preliminary data. We have devised and tested a centralized distribution mechanism at Stanford University of collating and shipping TMAs to participating sites, We have found shortcomings with the BLISS system and STMAD for histological image capture and storage for pathological review and have developed a much improved, highly efficient system using a Leica scanner and Path.XL image analysis software suite. We also have made significant progress in testing TACOMA, an automater TMA scoring algorithm. We have completed staining of the TMAs for H & E, High Molecular Weight Keratin, p27, ERG, SPKINKI, Ki67 (MIBI), MUCI, Survivin and PTEN FISH. Over the next year, we will expand our database to add more tested TMAS Biomarkers, perform QA/QC to ensure high quality, and evaluate their performance for predicting recurrence. We will further refine TACOMA algorithm to facilitate the scoring of TMA stains. We will work with investigators to write papers reporting tested TMA Biomarkers.

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TMA, Recurrence, biomarkers, Case-Cohort design, antibody

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Introduction

The most significant challenge in managing localized prostate cancer is the decision of whether or not it needs to be treated. Nearly ½ of prostate cancers diagnosed in the U.S. fall into the low or very low risk category and have little likelihood of causing death. However, it is well known that a significant fraction of low risk cases are misclassified and actually have occult high-risk features or are destined to progress to high-risk disease. Therefore a critical need in localized prostate cancer is the development of biomarkers that predict occult or incipient aggressive disease in the low-risk population.

To address this challenge, we formed the multi-institutional Canary Tissue Microarray Project. We have used rigorous clinical trial case/cohort design, taking care to correct for institutional and spectrum biases. Funding from the Department of Defense allowed us to complete construction of the TMAs as well as the necessary infrastructure and begin testing biomarker candidates. With this infrastructure in place, we have a robust validation platform for testing prostate cancer biomarkers. Based on our success, this resource will be a source for future biomarker validation studies even after the DOD funding has ceased.

The DOD has catalyzed the formation of the infrastructure to support this project and the study team have published 12 peer reviewed papers and reported our findings for nine biomarkers. Eleven additional biomarkers are under evaluations. Since the TMA resource we have established can evaluate hundreds of biomarkers, it is not possible to have all of them tested under this grant. Pathologist reading and scoring time is the limiting factor because the grant does not have resource to support that activities. Instead we rely on pathologists volunteering to do this due to their desire to validation their favorite biomarkers. I told the TMA study team that I am committed to analyze all their data as long as they read/score TMAs and send us the data. With the funding end now, the study team is still committed to complete the planned reading and evaluation of approved candidates. However, the reports will beyond the grant funding period. The team is also planning to submit additional grants support continuing activities.

My team as the MDACC will use my institutional resource to continue analyzing the upcoming data and helping authors to address comments from journal referees. Currently we are writing a paper integrating biomarkers that have been evaluated in this project and exam whether they complement to each other and whether an integrated model will improve the performance of predicting PCa recurrence.

Below we summarize the progress made for this grant.

Specific Aim 1) To test markers of prognosis on prostate cancer tissue microarrays with associated clinical data.
1.A. Develop work-flow for TMA sharing, image scanning, TMA staining data analysis.

The multi-institutional TMAs have been constructed at all sites. The final TMA cohort is 1326 patients with only 31 patients excluded due to data error. We are in the process of updating follow-up on the TMAs since several years of additional follow-up have been accumulated since the cases were first selected. Patients have been selected at random from the pool of patients who had undergone radical prostatectomy at each of the sites, with special attention to selecting patients with features typical of low-intermediate risk patients seen in contemporary urologic practices. Details of patient selection, statistical considerations, and TMA construction are

summarized in our publication in Advances in Anatomic Pathology published earlier this year and appended to last year's report. In addition to this cohort, a separate TMA has been constructed from 220 patients who underwent radical prostatectomy at a sister site who have very long term follow-up (up to 25 years) and hard endpoints including metastases and prostate cancer specific death. Since many of these patients were diagnosed in the pre-and early PSA eras, they are held separately as a validation cohort.

We have completed several stated aims in the proposal with regard to development of work-flow for array sharing, analysis and archiving while some aspects continue to be developed:

1) The Data Transfer Agreement (DTA) was completed between FHCRC and MDACC so the study data could be freely shared and communicated between FHCRC and MDACC. MDACC has established new database to warehouse the study data, receiving and archiving assay data from different labs/groups submitted to this project.

2) We have concluded that TACOMA algorithm as it currently stands, it is inadequate for automatic imaging reading. The main reason is that it still requires pathologists to sketch the boundary for cancer cell region. Though Dr. Tim Randolph will continue collaborating with Dr. Richard Levenson to add that functionality by another new software, it wouldn't be available in the life length of this project period to reduce pathologist reading time.

3) Data management and data analysis: We have performed data analyses for all biomarkers whose data has been submitted to MDACC. The details of the findings are summarized below.

1.B. Test candidate biomarkers of prognosis for prediction of recurrence after radical prostatectomy

In our ongoing monthly conference calls, the TMA investigators review progress and review applications for utilizing the TMAP resource. Most applications for use of the TMAs come from within the group, although it is available to the prostate cancer research community broadly and can be accessed by application through the Canary Foundation website (http://www.canaryfoundation.org). We have focused on biomarkers that have well characterized, highly performing reagents (e.g. immunohistochemical grade antibodies) and sufficient preliminary data that they could supply prognostic information independent of grade, stage and PSA. We have now completed staining for many of the biomarkers listed in our proposal and are expanding to novel biomarkers discovered since our application.

The primary objective is to correlate these two biomarkers with survival endpoints. Three survival endpoints were of interest: recurrence-free survival (RFS, where event was defined as any recurrence or metastasis or prostate cancer death), disease-specific survival (DSS, where event was defined as metastasis or prostate cancer death), and overall survival (OS, where event was defined as death of any cause).

We will first give an overall summary of the proposed assays and their status, followed by details of the findings not reported in previous progress report.

Summary of Proposed Assays and their Status

Applicant	Proposed Assay	Status
Squire/Troyer	PTEN FISH	Published
McKenney/Brooks	ERG	Published
McKenney/Brooks	SPINK1	Published
Lotan	PTEN-IF and PTEN-IHC	Both published
Tretiakova	Ki67	Published
McKenney	Histology patterns	Published
Brooks	AZGP1 in situ	Published
Brooks	AZGP1 antibody	Submitted
Brooks	MUC1	In press
Ayalo/McKenney	stromal quantification (H&E)	manuscript in prep
Brooks	ARG2 and CD38	analysis underway
Chatterjee	SULT2B	analysis of subset complete; additional proposal anticipated
Drake	N-glycan via MALDI	analysis of subset complete; additional proposal received
McKenney	Masson's trichrome	scoring underway
True	CD10	scoring underway
McKenney	p63	scoring underway
Brooks/Vakar-Lopez	p27	manuscript in prep
Brooks/Beck	HE4: prognostic model	on hold

Meng	MCM2	reviewed; conditional approval
Rohit Mehra	Schlap1	reviewed; conditional approval
Drake	N-glycan via MALDI and other	reviewed; not approved but revision requested
Liu	SMAD7	canceled; slides returned

Updates on completed biomarkers not reported in previous progress reports:

Objective: to identify a biomarker panel that significantly associated with recurrence-free survival in post-surgery prostate cancer patients. The data analyses are largely completed. The paper is under preparation to report the performance of biomarker panel in predicting prostate cancer recurrence.

Method:

Biomarker status was summarized using frequencies and percentages. Cox proportional hazard model stratified by site was used to correlate biomarkers with recurrence-free survival (RFS), where RFS event is defined as any recurrence, metastasis, or prostate cancer death post-surgery. Backwards elimination procedure starting with all biomarkers tested so far was used to identify the final model with only significant factors. All tests were two-sided and p-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC).

Table 1. Summary of patient characteristics and biomarker status.

	N	%
Margin		
Unknown	179	14.04
Positive	385	30.20
Negative	711	55.76
SVI		
Unknown	17	1.33
No	1177	92.31
Yes	81	6.35
ECE		
Unknown	17	1.33

Table 1. Summary of patient characteristics and biomarker status.		
	N	**%**
No	877	68.78
Yes	381	29.88
Gleason		
Unknown	10	0.78
<=6	549	43.06
3+4	458	35.92
4+3	143	11.22
8-10	115	9.02
PTEN(FISH)		
Unknown	663	52.00
Intact	500	39.22
Hemi-deletion	55	4.31
Homo-deletion	57	4.47
PTEN FISH		
Unknown	663	52.00
Any PTEN Deletion by FISH	112	8.78
PTEN Intact by FISH	500	39.22
PTEN(IHC)		

Table 1. Summary of patient characteristics and biomarker status.		
	N	**%**
Unknown	180	14.12
Intact	837	65.65
Hetero-loss	150	11.76
Homo-loss	108	8.47
PTEN(IHC)		
Unknown	180	14.12
Any PTEN Loss by IHC	258	20.24
PTEN Intact by IHC	837	65.65
Ki-67 Weighted Avg %Positive		
Unknown	271	21.25
High (>= 5%)	162	12.71
Low (< 5%)	842	66.04
ERG		
Unknown	245	19.22
Negative	604	47.37
Positive	426	33.41
SPINK		
Unknown	210	16.47

Table 1. Summary of patient characteristics and biomarker status.		
	N	**%**
Negative	1022	80.16
Positive	43	3.37
AZGP1(IHC)		
Unknown	139	10.90
Low	864	67.76
High	272	21.33
AZGP1(CISH)		
Unknown	186	14.59
Low	853	66.90
High	236	18.51
Stroma Index Number		
Unknown	218	17.10
Low (< 468.9)	741	58.12
High (>= 468.9)	316	24.78
Stroma Index Pct		
Unknown	215	16.86
Low (< 0.34)	795	62.35
High (>= 0.34)	265	20.78

Table 1. Summary of patient characteristics and biomarker status.		
	N	**%**
MUC1		
Unknown	95	7.45
Negative	865	67.84
Positive	315	24.71
MUC1		
Unknown	95	7.45
Negative	865	67.84
Weak	140	10.98
Intermediate	109	8.55
Strong	66	5.18
Histological Pattern Group		
N1 (any of Ex, Ey, Ez, Dy, Dz, Cy, Cz, Bz, Ay1 or Ay2 present)	376	29.49
N2 (N1 negative and Ew present)	3	0.24
N3 (N2 negative and Dx present)	113	8.86
N4 (N3 negative and Bx or Bz present)	135	10.59
N5 (N4 negative and Az present)	310	24.31
N6 (N5 negative and Bw, Cw, or Dw present)	36	2.82
N7 (None of above present)	302	23.69

Table 1. Summary of patient characteristics and biomarker status.		
	N	**%**
CD38(IHC)		
Unknown	170	13.33
Moderate/Strong	531	41.65
Negative/Weak	574	45.02
ARG2(IHC)		
Unknown	153	12.00
Negative	196	15.37
Weak	440	34.51
Intermediate	377	29.57
Strong	109	8.55
CD10		
Unknown	251	19.69
Negative	484	37.96
Positive	540	42.35
All	1275	100.00

	N	Mean	SD	Min	Median	Max
age	1169	61.45	7.09	35.00	62.00	80.00
Pre-op PSA	1147	8.61	8.29	0.02	6.40	124.00
p27HCytoMin	995	144.95	66.03	0.00	148.87	300.00
p27HNucMin	995	135.81	68.96	0.00	139.04	300.00
p27HCytoMax	995	186.45	64.16	0.19	198.79	300.00
p27HNucMax	995	180.85	66.94	0.15	191.88	300.00

Table 2. Summary of univariate Cox proportional hazard model results for recurrence-free survival (RFS), where RFS event is defined as any recurrence (biochemical, clinical, salvage therapy), metastasis, or prostate cancer death post-surgery. Hazard ratio higher than 1 means worse prognosis. Factors with 3 or more levels were assessed overall p-value first, pairwise p-values were only useful if overall p-value was significant (p<= 0.05). LCL = lower confidence limit, UCL = upper confidence limit

Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	Pairwise P-value	Overall P-value	#Event	#Censored	Total #Patients
Margin	Pos vs. Neg	2.0818	1.7442	2.4848	<.0001		495	601	1096
SVI	No vs. Yes	0.2956	0.2284	0.3826	<.0001		568	690	1258
ECE	No vs. Yes	0.5218	0.4413	0.6171	<.0001		571	687	1258
Gleason	3+4 vs. <=6	1.4339	1.1786	1.7444	0.0003	<.0001	570	695	1265
	4+3 vs. <=6	2.3891	1.8663	3.0584	<.0001				
	8-10 vs. <=6	2.3881	1.8205	3.1328	<.0001				
PTEN (FISH)	Intact vs. Homo-del	0.4342	0.3091	0.61	<.0001	<.0001	278	334	612
	Hemi-del vs. Homo-del	0.594	0.3662	0.9635	0.0348				
PTEN (FISH)	Any PTEN Deletion by FISH vs. PTEN Intact	1.7948	1.3643	2.3613	<.0001		278	334	612
PTEN (IHC)	Intact vs. Homo-loss	0.4909	0.3806	0.6331	<.0001	<.0001	505	590	1095
	Hetero-loss vs. Homo-loss	0.7021	0.511	0.9648	0.0292				
PTEN (IHC)	Any PTEN Loss by IHC vs. PTEN Intact by	1.6626	1.3744	2.0113	<.0001		505	590	1095
Ki-67 Weight Average %Pos	High vs. Low	1.8489	1.4805	2.3089	<.0001		450	554	1004
ERG	Neg vs. Pos	1.2058	1.0006	1.4532	0.0492		471	559	1030
SPINK	Neg vs. Pos	1.9688	1.11	3.4919	0.0205		490	575	1065
AZGP1 (IHC)	Neg vs. Pos	1.3603	1.1003	1.6818	0.0045		521	615	1136
AZGP1 (CISH)	Neg vs. Pos	1.2758	1.0206	1.595	0.0325		506	583	1089
stroma Index Num	Low vs. High	1	0.8225	1.2159	1		476	581	1057
stroma Index Pct	Low vs. High	0.8049	0.6586	0.9837	0.034		479	581	1060
MUC1	Neg vs. Pos	0.8097	0.6734	0.9737	0.0249		542	638	1180
Histological Pattern Group	N1 vs. N7	2.606	2.0478	3.3164	<.0001	<.0001	579	696	1275
	N2 vs. N7	3.0058	0.951	9.5004	0.0609				
	N3 vs. N7	1.7983	1.2898	2.5074	0.0005				
	N4 vs. N7	1.4446	1.0342	2.0178	0.031				
	N5 vs. N7	1.3676	1.0446	1.7904	0.0228				
	N6 vs. N7	0.7164	0.3477	1.4761	0.3659				

CD38 (IHC)	Moderate/Strong vs. Negative/Weak	0.7962	0.6682	0.9487	0.0108		507	598	1105
ARG2 (IHC)	Neg vs. Strong	0.8159	0.5831	1.1415	0.235	0.5661	514	608	1122
	Weak vs. Strong	0.819	0.6082	1.1029	0.1885				
	Intermediate vs. Strong	0.8766	0.649	1.1839	0.3902				
CD10	Neg vs. Pos	0.7869	0.6561	0.9437	0.0098		478	546	1024
Age	1 unit increase	1.0027	0.9912	1.0144	0.6465		559	610	1169
Log(Pre-op PSA)	1 unit increase	1.8724	1.6339	2.1457	<.0001		531	616	1147
p27HCytoMin	1 unit increase	0.9988	0.9975	1.0002	0.1057		451	544	995
p27HNucMin	1 unit increase	0.9985	0.9971	0.9998	0.0257		451	544	995
p27HCytoMax	1 unit increase	0.9993	0.9978	1.0007	0.3074		451	544	995
p27HNucMax	1 unit increase	0.9986	0.9972	0.9999	0.0415		451	544	995

Table 3a. Summary of multivariate Cox proportional hazard model for RFS. We used backwards elimination procedure started with all factors listed in table 2 and continued until only significant factors remained in the model. Hazard ratio higher than 1 means worse prognosis. Site was modeled as stratum. (N = 745, E = 335). Conclusions:

1. Positive margin, SVI, Gleason 4+3, histology groups N1-N5, any loss of PTEN on IHC, low stroma index number, and higher pre-op PSA were significantly associated with worse RFS after adjusting for each other's effects.
2. C-index = 0.67

Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
Margin	Pos vs. Neg	1.718	1.351	2.185	<.0001
SVI	Yes vs. No	1.619	1.124	2.332	0.01
Gleason	3+4 s. 6	1.056	0.797	1.399	0.71
	4+3 vs. 6	2.081	1.462	2.961	<.0001
	8-10 vs. 6	1.377	0.942	2.014	0.10
HistNG	N1 vs. N7	2.317	1.492	3.600	0.0002
	N2 vs. N7	2.210	0.642	7.605	0.21
	N3 vs. N7	1.795	1.088	2.961	0.02
	N4 vs. N7	1.845	1.119	3.042	0.02
	N5 vs. N7	1.623	1.042	2.527	0.03
	N6 vs. N7	1.042	0.398	2.730	0.93
	N1 vs. N5	1.428	1.059	1.926	0.02
PTEN (IHC)	Any loss vs. Intact	1.374	1.073	1.759	0.001
Stroma Index Number	<468.9 vs. >=468.9	1.324	1.035	1.695	0.01
Log(Pre-op PSA)	1 unit increase	1.335	1.118	1.594	0.03

Table 3b. Summary of another multivariate Cox proportional hazard model for RFS. We used backwards elimination procedure started with all factors listed in table 2 except for HistNG and PTEN FISH (more than 50% unknown), and continued until only significant factors remained in the model. Hazard ratio higher than 1 means worse prognosis. Model was stratified by site. Sample size (N = 736, E = 345) in this model is different from the one in table 3a. Conclusions:

1. Positive margin, SVI, ECE, Gleason score of 4+3, negative/weak AZGP1 (IHC), positive CD10, and higher pre-op PSA were significantly associated with worse RFS after adjusting for each other's effects.
2. C-index = 0.65

Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
Margin	Pos vs. Neg	1.650	1.297	2.099	<.0001
SVI	Yes vs. No	1.970	1.388	2.798	0.0002
ECE	Yes vs. No	1.428	1.113	1.833	0.005
Gleason	3+4 vs. 6	1.078	0.824	1.412	0.58
	4+3 vs. 6	1.728	1.224	2.438	0.002
	8-10 vs. 6	1.314	0.913	1.890	0.14
AZGP1(IHC)	Negative/Weak vs. Moderate/Strong	1.449	1.101	1.905	0.008
CD10	Pos vs. Neg	1.341	1.067	1.685	0.01
Log(Pre-op PSA)	1 unit increase	1.375	1.153	1.639	0.0004

Table 4. Summary of RFS status by Histology group. RFS event is defined as any recurrence, metastasis, or prostate cancer death post-surgery. The sample sizes of N2 and N6 were too small for modeling purpose.

	RFS Status	
	No Event	Event
	Number of Patients	Number of Patients
Histology Group		
N1	136	240
N2	0	3
N3	57	56
N4	80	55
N5	185	125
N6	28	8
N7	210	92

Table 5. Summary of multivariate Cox proportional hazard models for RFS by histology pattern group. Backwards elimination procedure was performed in each group separately until only significant factors remained. Hazard ratio higher than 1 means worse prognosis. Site was modeled as stratum. Conclusions:

1. Positive margin was significantly associated with worse RFS in N4, N5, and N7 groups. High pre-op PSA was significantly associated with worse RFS in N1, N3, and N7 groups. SVI was significantly associated with worse RFS in N1 and N5 groups.
2. High Ki-67 was significantly associated with worse RFS in N1 after being adjusted for SVI and pre-op PSA.

Histology Group	Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value	Total Number of Patients	Number of Events
N1	SVI	Yes vs. No	1.716	1.135	2.596	0.01	266	176
	Ki-67	>=5% vs. <5%	1.510	1.082	2.108	0.02		
	log(PSA)	1 unit increase	1.755	1.390	2.215	<.0001		
N3	log(PSA)	1 unit increase	2.103	1.356	3.260	0.0009	106	55
N4	Margin	Pos. vs. Neg.	2.644	1.375	5.082	0.004	116	48
N5	Margin	Pos. vs. Neg.	2.412	1.621	3.587	<.0001	265	109
	SVI	Yes vs. No	3.076	1.507	6.280	0.002		
N7	Margin	Pos. vs. Neg.	2.078	1.229	3.515	0.006	224	70
	log(PSA)	1 unit increase	1.543	1.057	2.250	0.02		

Table 6a. Summary of final multivariate model for RFS after multiple imputation and weighting with HistNG in the model. Twenty imputations were carried out to produce 20 full data sets (N = 1275, E = 579). Each observation was weighted by its inverse sampling probability and inverse probability of censoring weight (IPCW). IPCW was calculated using method of Cole (2004). Backwards elimination procedure was carried out using p-values from combining inferences from 20 models (Rubin, 1987). We started from full model with all biomarkers and clinical factors. Cox model was fit to each imputation data set, and estimates of coefficients were obtained. Pooled inference over 20 models was performed and the least significant factor was eliminated. The reduced model was fit to all 20 imputation data sets and inference and elimination continued. This process was repeated until only significant factors remained. Site was modeled as stratum. Conclusion:

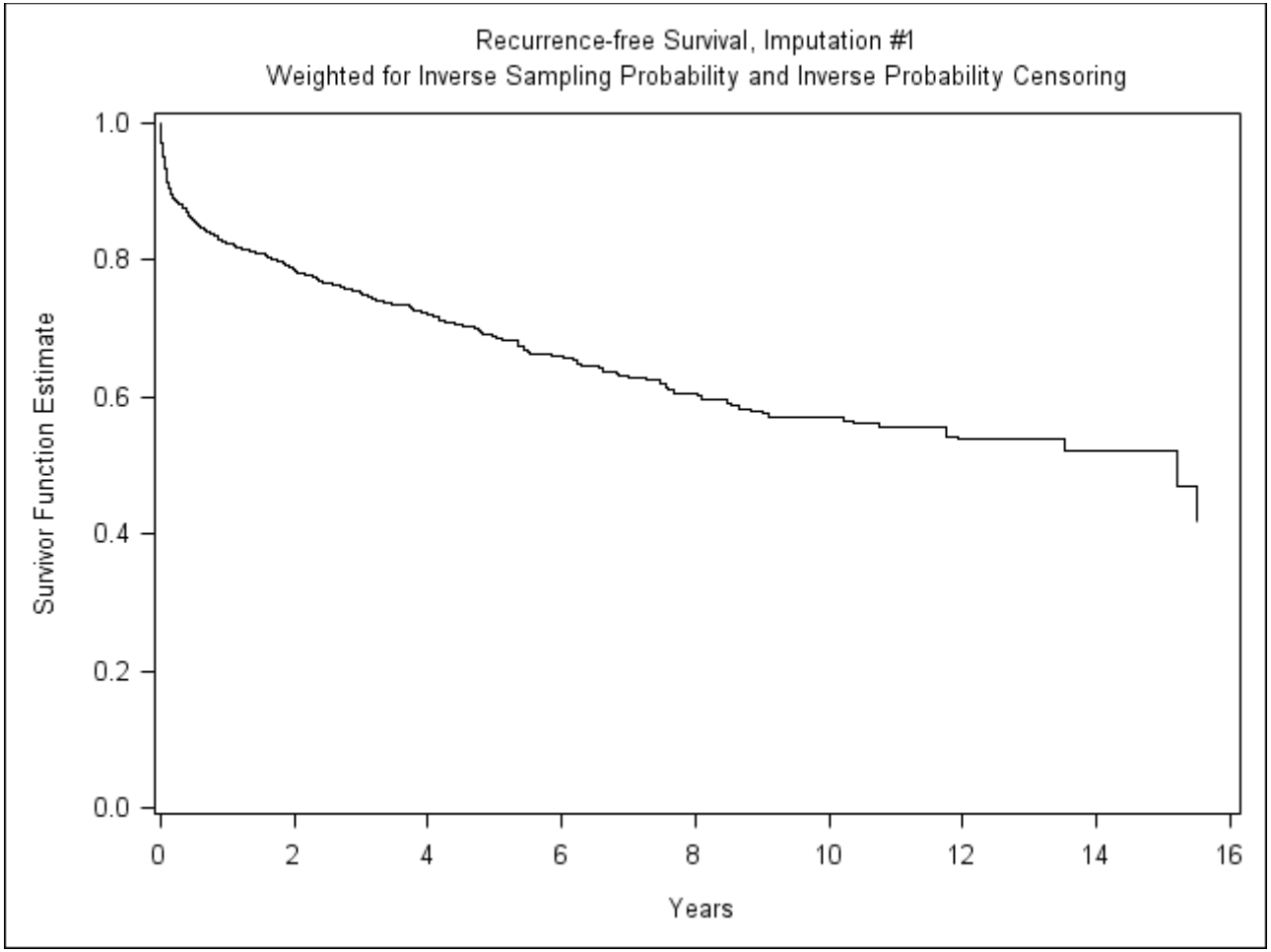
1. Positive margin, SVI, any loss of PTEN by IHC, HistNG N1 and N4, and higher pre-op PSA were significantly associated with worse RFS.

Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
Margin	Pos vs. Neg	1.885	1.403	2.532	<.0001
SVI	Yes vs. No	2.215	1.575	3.115	<.0001
PTEN(IHC)	Any Loss vs. No	1.602	1.240	2.071	0.0003
HistNG	N1 vs. N7	1.818	1.330	2.485	0.0002
	N2 vs. N7	1.277	0.224	7.296	0.78
	N3 vs. N7	1.312	0.859	2.005	0.21
	N4 vs. N7	1.656	1.131	2.425	0.01
	N5 vs. N7	1.348	0.976	1.862	0.07
	N6 vs. N7	0.497	0.198	1.248	0.14
Log(pre-op PSA)	1 unit increase	1.430	1.201	1.704	<.0001

Table 6b. Summary of final multivariate model for RFS after multiple imputation and weighting without HistNG in the model. Twenty imputations were carried out to produce 20 full data sets (N = 1275, E = 579). Each observation was weighted by its inverse sampling probability and inverse probability of censoring weight (IPCW). IPCW was calculated using method of Cole (2004). Backwards elimination procedure was carried out using p-values of combining inferences from 20 models (Rubin, 1987). We started from full model with all biomarkers and clinical factors. Cox model was fit to each imputation data set, and estimates of coefficients were obtained. Pooled inference over 20 models was performed and the least significant factor was eliminated. The reduced model was fit to all 20 imputation data and inference and elimination continued. This process was repeated until only significant factors remained. Site was modeled as stratum. Conclusion:

1. Positive margin, SVI, any PTEN loss by IHC, and higher pre-op PSA were significantly associated with worse RFS.

Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
Margin	Pos vs. Neg	1.853	1.379	2.490	<.0001
SVI	Yes vs. No	2.270	1.605	3.211	<.0001
PTEN(IHC)	Any Loss vs. No	1.748	1.359	2.248	<.0001
Log(pre-op PSA)	1 unit increase	1.433	1.204	1.704	<.0001


Recurrence-free Survival, Imputation #1
Weighted for Inverse Sampling Probability and Inverse Probability Censoring
Survivor Function Estimate
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
Years

References:

Rubin, D. B. (1987), *Multiple Imputation for Nonresponse in Surveys*, New York: John Wiley & Sons.

Cole SR, Hernan MA. Adjusted survival curves with inverse probability weights. *Comput. Methods Programs Biomed*. [electronic article]. 2004;75(1):45–49.

Specific Aim 2) To evaluate candidate markers that correlate with Gleason grade on prostate cancer tissue microarrays with associated clinical data.

Thus far, we have focused on building the analysis pipeline and in staining high priority biomarkers of prognosis. In all of the biomarkers we have tested thus far, we have interrogated each for its correlation with Gleason score. In general, most of them are correlated, although not completely. While these do not address the intent of this Aim, we are not disappointed since it does appear that *these biomarkers are supplying prognostic information that is independent of Gleason score.* The intent of Aim 2, on the other hand, was to investigate biomarkers that correlate with Gleason grade. Several markers are in our queue and are listed in the original proposal. For some, we are still looking for high quality affinity reagents that provide interpretable staining with limited background. Leading candidates are AGR2, a marker expressed at high levels in Gleason pattern 3 cancers and Monoamine oxidase A, expressed at high levels in Gleason pattern 4 disease. As we get through our candidate prognostic markers (listed above and in the queue) we will refocus on biomarkers that predict Gleason grade. This could be useful in characterizing biopsy samples to predict upgrading.

However, this clinical question might become less relevant in the future since several tools have been developed that already predict up-grading. For example the OncotypeDx assay has been calibrated and already validated precisely for this purpose. In addition, multiparametric MRI shows good correlation with grade in that only the high- grade lesions are visible, while the low grade lesions are not. As the clinical practice evolves, we will decide whether we wish to continue to pursue development of IHC biomarkers that predict Gleason score

For all biomarkers, whether for Gleason score or prognosis, the statistical analysis strategy has been outlined in our proposal and will be used as soon as reads are available from the pathologists, both in their correlations with Gleason score and in their complementary property with Gleason score.

Key Research Accomplishments

- Provided statistical expertise in biomarker review and approval by the investigative team to ensure quality of the reagents and sufficient level of evidence for investigation of a particular biomarker on our valuable resource.
- Data receiving, reconcile data questions, and archiving at MDACC.
- Received final clinical data that will be used for analysis of biomarker performance to the MD Anderson DMCC.
- Established and tested the data analysis pipeline for anticipated additional biomarker data.
- Evaluated TACOMA imaging analysis algorithm using Survivin, CD117, and ERG data and concluded that it is inadequate for automated imaging analysis as it stands along.
- Completion of analysis of PTEN FISH and a manuscript published.
- Completion of analysis of Ki67 PI and a manuscript published.
- Completion of analysis of ERG IHC and PTEN IHC and presentation at international meetings and results published.
- Completion of analysis of SPINK and results published.
- Completion of analysis of AZGP1 results published.
- Completion of analysis of a modified Gleason grading system with Jesse McKenney, manuscript published.
- Completion of analysis of Muc1 and results published.
- Ongoing analysis of stromal quantification, ARG2, CD38, SULT2B, N-glycan via MALDI, Masson's trichrome, CD10, p63, and p27. We expect all of these, regardless of outcome (prognostic or not) will be submitted as separate publications.
- Significant preliminary data from this collaboration that will position us well for the next phase of funding.

Reportable Outcomes

1) Publications referencing this grant:

James D. Brooks: Translational genomics: The challenge of developing cancer diagnostic biomarkers. *Genome Research* **22:** 183-187, 2012.

Sarah Hawley, Ladan Fazli, Jesse K. McKenney, Jeff Simko, Dean Troyer, Marlo Nicolas, Lisa F. Newcomb, Janet E. Cowan, Luis Crouch, Michelle Ferrari, Javier Hernandez, Antonio Hurtado-Coll, Kyle Kuchinsky, Janet Liew, Rosario Mendez-Meza, Elizabeth Smith, Imelda Tenggarra, Xiaotun Zhang, Peter R. Carroll, June M. Chan, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, Ziding Feng, Lawrence D. True and James D. Brooks: Design and construction of a resource for the validation of candidate prognostic biomarkers: the Canary Prostate Cancer Tissue Microarray as a model. *Advances in Anatomic Pathology* **20:** 39-44, 2013.

J James D. Brooks: Managing localized prostate cancer in the era of prostate specific antigen testing. *Cancer* **119:** 3906-3909, 2013.

Zuxiong Chen, Zulfiqar G. Gulzar, Catherine A. St. Hill, Bruce Walcheck, James D. Brooks: Increased expression of *GCNT1* is associated with altered *O*-glycosylation of PSA, PAP and MUC1 in human prostate cancers. *Prostate* **74:** 1059-1067, 2014.

Troyer D, Jamaspishvili T, Wei W, Feng Z, Good J, Hawley S, Fazli L, McKenney J, Simko J, Hurtado-Coll A, Carroll P, Gleave M, Lance R, Lin D, Nelson P, Thompson I, True L, Brooks J, Squire J. A multicenter study shos PTEN deletion is strongly associated with seminal vesicle involvement and extracapsular extension in localized prostate cancer. The Prostate. 75(11): 1206-1215, 2015.

James D. Brooks, Wei Wei, Sarah Hawley, Heidi Auman, Lisa Newcomb, Hilary Boyer, Ladan Fazli, Jeff Simko, Antonio Hurtado-Coll, Dean A. Troyer, Peter R. Carroll, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, Lawrence D. True, Ziding Feng and Jesse K. McKenney. Evaluation of ERG and SPINK1 by immunohistochemical staining and clinicopathological outcomes in a multi-institutional radical prostatectomy cohort of 1067 patients. PLOS ONE 10(7): e0132343. Doi:10.137/journal.pone.0132343.

TL Lotan, W Wei, CL Morais, ST Hawley, L Fazli, A Hurtado-Coll, D Troyer, JK McKenney, J Simko, PR Carroll, M Gleave, R Lance, DW Lin, PS Nelson, IM Thompson, LD True, Z Feng, JD Brooks, PTEN loss as determined by clinical-grade immunohistochemistry assay is associated with worse recurrence-free survival in prostate cancer, **2016**, *European Urology Focus*, 2(2): 180-188

MS Tretiakova, W Wei, HD Boyer, LF Newcomb, S Hawley, H Auman, F Vakar-Lopez, JK McKenney, L Fazli, J Simko, DA Troyer, A Hurtado-Coll, IM Thompson Jr., PR Carroll, WJ Ellis, ME Gleave, PS Nelson, DW Lin, LD True, Z Feng, JD Brooks, Prognostic value of Ki67 in

localized prostate carcinoma: a multi-institutional study of >1,000 prostatectomies, **2016**, *Prostate Cancer and Prostatic Diseases*, 19(3): 264-270

TL Lotan, W Wei, O Ludkovski, CL Morais, LB Guedes, T Jamaspishvili, K Lopez, ST Hawley, Z Feng, L Fazli, A Hurtado-Coll, JK McKenney, J Simko, PR Carroll, M Gleave, DW Lin, PS Nelson, IM Thompson, LD True, JD Brooks, R Lance, DA Troyer, JS Squire, Analytic validation of a clinical-grade PTEN immunohistochemistry assay in prostate cancer by comparison with PTEN FISH, **2016**, *Modern Pathology*, 29(8): 904-914

JD Brooks, W Wei, JR Pollack, RB West, J Sunwoo, JH Shin, SJ Hawley, H Auman, LF Newcomb, J Simko, A Hurtado-Coll, DA Troyer, PR Carroll, ME Gleave, DW Lin, PS Nelson, IM Thompson, LD True, JK McKenney, Z Feng, L Fazli, Loss of expression of AZGP1 is associated with worse clinical outcomes in a multi-institutional radical prostatectomy cohort, **2016**, *Prostate*, 76(15): 1409-1419

JK McKenny, W Wei, S Hawley, H Auman, LF Newcomb, HD Boyer, L Fazli, J Simko, A Hurtado-Coll, DA Troyer, MS Trestiakova, F Vakar-Lopez, PR Carroll, MR Cooperberg, ME Gleave, RS Lance, DW Lin, PS Nelson, IM Thompson, LD True, Z Feng, JD Brooks, Histologic grading of prostatic adenocarcinoma can be further optimized: analysis of the relative prognostic strength of individual architectural patterns in 1275 patients from the Canary retrospective cohort, **2016**, *Am. J. Surg. Pathol.*, 40(11): 1439-1456

O Eminaga, W Wei, SJ Hawley, H Auman, LF Newcomb, JSimko, A Hurtado-Coll, DA Troyer, PR Carroll, ME Gleave, DW Lin, PS Nelson, IM Thompson, LD True, JK McKenney, Z Feng, L Fazli, JD Brooks, MUC1 Expression by Immunohistochemistry is Associated with Adverse Pathologic Features in Prostate Cancer: A Multi-Institutional Study, **2016**, *PLoS One*, (in press)

Conclusion

We have undertaken a challenging task of creating a multi-institutional TMA resource with rigorous case/cohort design. To our knowledge, such a resource has not been previously created and offers the advantage of reducing institutional biases as well as spectrum biases. In the uniform design and through image acquisition and archiving technologies, we have created a resource that can be easily used by the greater prostate cancer research community. In many ways, this resource represents a gold standard for evaluation of prognostic biomarkers. We have completed all phases of pipeline construction and continue to refine our work-flow to improve functionality as we work with the resource. We now have tested several biomarkers and confirmed that they are prognostic, and resulted 9 peer reviewed publications. In addition, even now this grant is to the end, the team and the infrastructure we have created will continue because the team is committed to continue validation promising biomarkers until our TMAs have been depleted. We will continue to carry out analysis of new biomarkers and solicit applications for biomarkers inside and outside our research group. This research directly addresses the PCRP overarching challenge to *distinguish lethal from indolent disease*.

List of personnel (not salaries) receiving pay from the research effort

Ziding Feng, Ph.D.,
Tim Randolph, Ph.D.,
Deanna Stelling
Jaime Henne

Wei Wei
Francesco Stingo
Aron Joon
Qingxiang Yan
Leonidas Bantis

Perspective

Translational genomics: The challenge of developing cancer biomarkers

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Early detection and definitive treatment of cancer have been shown to decrease death and suffering in epidemiologic and intervention studies. Application of genomic approaches to many malignancies has produced thousands of candidate biomarkers for detection and prognostication, yet very few have become established in clinical practice. Fundamental issues related to tumor heterogeneity, cancer progression, natural history, and biomarker performance have provided challenges to biomarker development. Technical issues in biomarker assay detection limits, specificity, clinical deployment, and regulation have also slowed progress. The recent emergence of biomarkers and molecular imaging strategies for treatment selection and monitoring demonstrates the promise of cancer biomarkers. Organized efforts by interdisciplinary teams will spur progress in cancer diagnostics.

Since the war on cancer was declared in 1972, cancer death rates, after rising for several decades, have begun to slowly decline (Siegel et al. 2011). This drop can be attributed to preventive efforts (e.g., smoking cessation), improved treatments for advanced disease, and early detection and treatment of localized cancers. Future progress in prevention and treatment of advanced disease requires a fundamental understanding of the underlying causes and mechanisms of cancer. Early detection, on the other hand, can be agnostic as to cause, and merely requires a method (imaging, cell collection, measurement of a bioanalyte) that correlates with a disease state, followed by the application of localized treatments (surgery, radiation, or tissue ablation) that have been developed and refined over the past century. Randomized trials of early detection and definitive local treatment have demonstrated improved survival for breast, colon, prostate, and lung cancers (Glass et al. 2007; Levin et al. 2008; Schroder et al. 2009; National Lung Screening Trial Research Team 2011). The results from these trials suggest that development of cancer diagnostic biomarkers is a desirable and attainable goal in the struggle to decrease deaths from cancer. Yet, why has progress been so slow, and where are the new cancer diagnostic biomarkers? Nearly all of the cancer biomarkers currently used in the clinic, such as prostate specific antigen (KLK3) or PSA (prostate cancer), ERBB2 (breast cancer), MUC16 (also known as CA-125) (ovarian cancer), alpha-fetoprotein (AFP), and beta-human chorionic gonadotropin (CGB) (testicular cancer), were discovered serendipitously. The advent of discovery-based approaches, such as array-based detection of gene expression and proteomic approaches using mass spectrometry, seemingly opened a fire hose of candidate cancer biomarkers over the last decade (McDermott et al. 2011). Relatively complete cataloguing of the genomic alterations for most major malignancies is now under way using rapidly evolving ultra-high-throughput sequencing approaches in large international consortia including The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium. Therefore, there should be no shortage of new biomarkers available for clinical translation. A search of the literature for the term "cancer biomarker" results in thousands of candidates, and many have been tested on clinical samples and show some utility. There the story usually stops (Ludwig and Weinstein 2005; Ioannidis and Panagiotou 2011). In fact, the number of biomarkers approved by the FDA each year for clinical use is in the single digits. This drop-off rate shows that the development of biomarkers is every bit as difficult as the development and approval of a new drug.

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Features of biomarkers that affect their performance

Many factors contribute to the failure of candidate biomarkers to realize clinical utility. The ideal cancer detection biomarker should be found uniquely in the malignant tissue and should generate a positive signal that can be measured without confounding noise from normal tissues or other non-malignant pathologies. PSA, for instance, has been criticized because it can be elevated by a variety of pathologies, making the positive predictive value (the chance that a man with an elevated PSA has prostate cancer) quite low, at 20%–40%. Therefore, many men undergo unnecessary biopsies with attendant side effects. An ideal test would limit false-positive tests, while enriching for cancers that are more likely to be lethal.

While gene expression profiling has revealed hundreds or even thousands of genes expressed at higher levels in malignant compared with benign tissues, virtually no transcripts or proteins have been identified that are uniquely elevated in cancer. Many candidate biomarkers belong to pathways intrinsic to normal cells and tissues, such as those mediating proliferation, apoptosis, differentiation, angiogenesis, cell death, and inflammation (Hanahan and Weinberg 2011). Some biomarkers have failed because their cognate protein levels, which are the preferred analyte for most clinical assays, do not correlate with transcript levels. Other candidate transcripts or proteins show only a relative increase in expression in cancer compared with normal tissue and therefore fail as biomarkers because low-level expression from the parent normal tissue, from other organ sites, or from non-malignant pathologies effectively drowns out the signal from the malignancy. Candidate biomarkers expressed in the nucleus or cytoplasm are not accessible to clinical assays since most biomarkers currently in use are cell surface or secreted proteins.

One proposed way to limit false-positive tests is to limit screening to a high-risk population based on identified germline "high-risk" alleles. Unfortunately, large genome-wide association studies in several malignancies have thus far identified alleles that

confer only small, clinically insignificant increases in cancer risk (Garcia-Closas et al. 2008; Kiemeney et al. 2008; Zheng et al. 2008; Song et al. 2009). Assays of these SNPs either singly or in groups often contribute little to risk assessment beyond asking a patient whether they have a family history for a particular malignancy (Zheng et al. 2008). Of course, there are notable exceptions, including *BRCA1* and *BRCA2*, *VHL*, *TP53*, and other germline mutations. But as a class, these hereditary cancer syndromes constitute only a small fraction of patients in a population and usually occur in clinically recognizable familial clusters. Therefore, tests that assay either rare or low-risk alleles are not useful for screening a population.

Somatically acquired alterations in cancer DNA show potential for detection since they are unique to the cancer and detectable with available technologies. In serous ovarian cancer, for example, TCGA has recently reported that 96% of cancers possess a mutated *TP53* gene (The Cancer Genome Atlas Research Network 2011). Point mutations in specific oncogenes have been identified in many other malignancies, such as *KRAS* mutations in colon cancer, and diagnostic tests have been designed to detect these mutations non-invasively (Dong et al. 2001). Cancer-specific hypermethylation of cytidine residues in CpG islands is common, and diagnostic tests to detect methylation at specific loci have been developed for several malignancies (Hoque et al. 2004, 2005, 2006; Topaloglu et al. 2004; Chen et al. 2005). Recent genome-wide methylation studies suggest that there may be many such methylation events and many biomarker candidates (Houshdaran et al. 2010; Kobayashi et al. 2011). Fusion transcripts, such as *TMPRSS2-ERG* in prostate cancer, represent another promising source of cancer-specific biomarkers (Tomlins et al. 2005). A urine-based test for *TMPRSS2-ERG* transcripts has been developed and is currently under evaluation in several patient cohorts (Salami et al. 2011).

While these and other avenues are promising, comprehensive analyses of cancer genomes have revealed a great deal of heterogeneity in the spectrum of mutations and structural alterations within cancers of a single histologic type. Ovarian cancers show startling variation in DNA structural changes between tumors at more than 100 loci throughout the genome (The Cancer Genome Atlas Research Network 2011). In glioblastomas, genomic alterations are restricted to a few discrete pathways, yet the frequency of alterations for any single member of those pathways is relatively low, making it difficult to design strategies for detection (The Cancer Genome Atlas Research Network 2008). In prostate cancer, only half of tumors have *TMPRSS2-ERG* fusion transcripts, and no frequently occurring point mutations have been identified (Tomlins et al. 2005; Berger et al. 2011). Therefore, in virtually all malignancies, assays of any single structural alteration or mutation will identify only a fraction of prevalent cancers, necessitating development of multiplex assays to interrogate entire pathways or many chromosome loci. Even in cases in which a single gene is inactivated by mutation, such as *TP53* in ovarian cancer, cost-effective assays will have to be developed for detection of the spectrum of mutations occurring in the gene. Furthermore, these detection strategies also must allow sensitive detection of mutated sequences against a background of wild-type sequences that are found in any clinical sample (e.g., blood or urine).

Biomarker challenges in the context of cancer biology

To be effective, a screening strategy must detect malignant cells that are destined to grow, metastasize, and cause death. Unfortunately, little is known about the steps that lead transformed cells to become malignant and ultimately lethal, and this has major implications for biomarker performance. Cancers are complex tissues composed of many cell types. It is possible (and likely) that features of the host, such as the innate immune response to the malignancy, interactions of the malignant cells with the surrounding stroma, or stochastic factors that are not captured by any biomarker, are important in the progression of early lesions (Chaffer and Weinberg 2011). Therefore, focusing on mutations or structural alterations within the malignant cells alone will be of limited utility in predicting biological and clinical behavior. Cancers possess heterogeneous populations of malignant cells including small populations of cancer stem cells that might be the source of metastatic cells (Reya et al. 2001). Biomarkers developed against the bulk mass of the tumor could miss the attributes of the stem cells that ultimately determine the clinical course of a malignancy. In addition, many biomarkers fail because most malignancies display genomic instability and require multiple genetic hits to become metastatic (Liu et al. 2009; Shah et al. 2009; Stephens et al. 2011). Measurement of a biomarker at a particular time might not predict acquisition of those future genetic alterations that are the product of this underlying genomic instability.

Performance of a diagnostic biomarker will be influenced by the natural history of the malignancy. Many cancers take years or even decades before they progress to lethality, suggesting that there might be a large window of opportunity for detection and eradication of transformed cells. In pancreatic cancer, for instance, high-throughput sequencing of primary and metastatic tumors suggests that 15–20 years transpire between initiation and cancer death (Yachida et al. 2010). In breast and prostate cancer, that window might be even longer (Liu et al. 2009; Shah et al. 2009). In current clinical practice using standard histology, however, that window is much shorter. For example, in serous ovarian cancer, there appears to be a 4-yr window from the moment cancer cells become histologically identifiable until they become metastatic (Brown and Palmer 2009). While this window appears to provide ample time for detection, the average size of these cancers at the time of metastasis is 9 mm in diameter. More importantly, to produce a 50% decrease in ovarian cancer mortality, it is estimated that tumors would need to be detected when they are 5 mm in diameter. Even if an ovarian cancer-specific protein is identified (one has not), detection of that protein from a 5-mm tumor diluted in a 5-L blood volume of a 60-kg woman is well beyond the sensitivity of currently available technologies.

Further complicating cancer detection strategies are genetic and histologic changes that occur with aging. Autopsy studies show histologically identifiable cancer precursor lesions (dysplasias and frank neoplasias) in a high proportion of the population and in many organ sites (Henson and Albores-Saavedra 2001). This leads to a fundamental problem in cancer detection: If a biomarker is able to detect these initiated lesions of which only a fraction will progress, how does one sort out which lesions need to be treated and which can be safely ignored? Inability to predict future cancer behavior (or prognosis) will inevitably lead to overtreatment. For prostate cancer, data from the European Randomized Study of Screening for Prostate Cancer suggested that 48 PSA screen-detected men must undergo surgery to save one man's life 9 yr after treatment (Schroder et al. 2009). Since prostate cancer treatments have life-altering consequences and financial costs, the U.S. Preventive Task Force has recently recommended against PSA screening since it leads to overtreatment of indolent prostate cancers (Sanda et al. 2008; Barry 2009). Undoubtedly, overdetection and overtreatment

will occur in other malignancies as tests to detect early disease are developed (Bach et al. 2007). Therefore, successful development of a biomarker for cancer detection must be coupled with development of biomarkers of prognosis to avoid overtreatment of clinically indolent cancers.

Challenges in developing and commercializing biomarker assays

Until recently, measurement of protein biomarkers has been constrained by the limits of detection of ELISA assays (usually in the nanogram per milliliter range) or by the signal that can be generated with imaging approaches that exploit metabolic pathways (such as 18F-FDG-PET) or use affinity reagents such as tagged antibodies (Gao et al. 2011). Several clever strategies using microfluidic methods and nanofabrication have been devised for affinity detection, often with several orders of magnitude increases in sensitivity (Gaster et al. 2009). However, both imaging and nanodetection strategies rely on high-quality affinity reagents–predominantly antibodies. Unfortunately, clinical translation of many candidate biomarkers is often stalled by the lack of well-characterized, highly specific monoclonal antibodies. Several biomarker assays are based on the detection of nucleic acids, such as the *PCA3* test for prostate cancer (which entails detection of a noncoding RNA in the voided urine) and the Oncotype DX test (which assays a panel of transcripts in breast cancer tissue samples) (Lee et al. 2011; Tang et al. 2011). Each of these tests requires special handling of samples because of endogenous nucleases. These special handling procedures can limit dissemination of biomarker assays because practice patterns do not accommodate them.

Economic and business considerations can slow cancer detection biomarker development. Many of the diagnostic and prognostic markers that have been reported in the literature are in the public domain and lack intellectual property protection. Companies have shied away from developing clinical tests absent those protections. Even in cases in which an assay is protected, companies face challenges in developing a clinical-grade assay with performance characteristics in reproducibility and accuracy that far exceed academic research standards. Assays have to meet stringent performance criteria regulated by the Clinical Laboratory Improvement Amendment (CLIA) under the Centers for Disease Control (CDC). FDA approval of a clinical diagnostic requires scrutiny of the assay and demonstration of effectiveness in affecting clinical decision-making in ways that favorably impact health. With the advent of evidence-based medicine, the standards for efficacy and approval have become more stringent. Cancer detection approaches need to show improved patient outcomes in morbidity or mortality, necessitating large, costly randomized clinical trials. The difficulty in designing, carrying out, and funding these types of trials has been highlighted in the failure of recent studies of "established" screening strategies (PSA screening and mammograms) to show patient benefit (Andriole et al. 2009; Gotzsche and Nielsen 2011). To complicate matters, rules governing traditional clinical assays are in the process of being rewritten as new, multiplexed assays are being brought forward for approval, leading to additional delays that can be costly for industry.

Despite these challenges, favorable markets have emerged for several cancer biomarkers. Most of these new biomarkers target treatment selection, and most require tumor biopsy samples in order to be performed. Biomarker assays that predict tumor aggressiveness have been developed for breast and colon cancers (Oncotype DX and MammoPrint) and are being used in clinical practice to select patients for adjuvant chemotherapy in early-stage disease. Sequencing for *EGFR* mutations in lung cancer identifies a relatively small set of cases (5% of lung adenocarcinomas) that will respond (often dramatically) to the EGFR inhibitor gefitinib (Iressa) (Paez et al. 2004). In fact, this discovery rescued gefitinib after clinical trials showed a lack of effectiveness in unselected lung cancer patients and has demonstrated the utility of using biomarkers in new drug development (Giaccone et al. 2004; Herbst et al. 2004; Wang et al. 2011). By using biomarkers for rational selection of patients for cancer clinical trials, companies improve their chances of success and thereby speed the drug approval process. In patients with Gastrointestinal Stromal Tumors (GISTs), response to therapy can be interrogated within days after administration of imatinib (Gleevec) by using 18F-FDG-PET imaging (Gayed et al. 2004). Many molecular imaging approaches are now under investigation for similar uses in other cancers. Use of molecular imaging or biomarkers to show lack of treatment efficacy allows patients and physicians to quickly abandon futile therapies in favor of other potentially effective treatments. While the clinical goals and measurement approaches for these types of trials differ in many ways from those required for early detection, successful application of molecular imaging and biomarkers for treatment selection and monitoring will lay important groundwork for future progress in cancer detection.

Clinical considerations in biomarker development

Discovery-based genomic studies have relied on samples of convenience—namely, tissue samples that have been banked from surgeries on individuals with relatively advanced disease. For example, virtually all of the serous ovarian cancer samples analyzed in TCGA were harvested from women with advanced (Stages III and IV) tumors (The Cancer Genome Atlas Research Network 2011). The number of genomic changes in these advanced cancers is extraordinary, making it difficult to identify critical early changes that could be used as diagnostic biomarkers. To be meaningful in a screened population, diagnostic biomarkers must be discovered in early-stage, non-metastatic cancers since biomarker expression can change over the course of a disease. In prostate cancer, for example, PSA expression per cancer cell usually decreases as tumors become dedifferentiated and metastatic, making PSA an unreliable predictor of therapeutic response in late stages of the disease (Eisenberger and Nelson 1996).

The use of convenience samples can also affect the performance of prognostic biomarkers, leading to subsequent failure to validate the biomarker in another patient population (Ioannidis and Panagiotou 2011). Quite commonly, cancer prognostic biomarkers are tested in patient samples from cases with early treatment failure or death, and these are compared with cases without recurrence many years after their treatment. This design, however, pits the worst cases against the very best. Clinical practice encompasses patients with a spectrum of risk, and biomarkers developed on samples from the tails of the bell-shaped curve are destined to fail. Therefore, development and validation of biomarkers need to be performed in the context of a discretely defined clinical question, with appropriately selected patients and adequate statistical power.

The behavior of a screening biomarker will also be influenced by how frequently the malignancy occurs in a population of individuals. Because the incident rates for any single cancer are fairly low in the population, any screening tool must display relatively high sensitivity (the portion of cancer cases that have a positive

test) and specificity (the portion of individuals without cancer that have a negative test). Tests with poor performance characteristics can miss cases (false negatives) or identify individuals as harboring the disease when they do not (false positives). False-negative tests have obvious consequences, since dangerous cancers will go undiagnosed. False-positive tests will lead to ancillary tests, such as imaging, laparoscopy, or biopsy, and thereby produce considerable costs to the patient, who suffers from anxiety and from the morbidity of those ancillary tests. False-positive tests and ancillary testing also incur significant financial costs to the patient and the health care system. The potential for harm is great, and this necessitates development of tests with high sensitivities and specificities. For instance, ovarian cancer occurs at a rate of 10–14 cases per 100,000 women per year. If one sets a relatively low bar for an ovarian cancer screening test (e.g., a positive predictive value of 10%, meaning that 10% of women with a positive test will have ovarian cancer, while 90% will have a false-positive test), the test specificity must exceed 99%, even for sensitivity values at 80% (i.e., 20% of cases would be missed by the test) (Lutz et al. 2011). These performance characteristics are well beyond most biomarkers currently in use or in development. Application of a screening test to a population raises additional questions including when to start screening, how frequently to screen, which population to screen, and how well the test performs in different ethnic populations.

A final challenge in clinical application of cancer biomarkers is with the end-users—the physicians who order the tests. Clinicians tend to use cancer screening tests in a binary fashion: A test is normal or abnormal based on whether it exceeds a cut-off value. However, emerging data suggest that existing cancer biomarkers should be used to assess risk as continuous variables, much as cholesterol is used to assess cardiovascular risk (Thompson et al. 2004). Several genomics-based tests, such as Oncotype Dx and MammaPrint in breast cancer, provide a risk score that correlates with a meaningful clinical outcome such as the subsequent chance of developing metastases. Yet, discussion of the relative risks of a quantitative test outcome requires considerable time, often a scarce resource in a busy practice. These discussions also require high levels of sophistication since patients will usually ask questions about steps downstream from the diagnosis of cancer that require an understanding of tumor aggressiveness and choice of therapy. How to deliver this information to patients compassionately, thoroughly, and accurately in the context of the current care delivery system and in the context of our limited understanding of cancer biology is a significant challenge.

The future

Development of cancer detection biomarkers will be propelled by technological improvements in how biomarkers are objectively measured (mutations, methylation, protein expression, molecular imaging). For example, as ultra-high-throughput sequencing technology improves and becomes more cost-effective, whole genome sequencing of germline DNA could identify rare, highly penetrant, high-risk alleles for many cancers that can be used to tailor cancer screening protocols to individuals at high risk. Development of robust sequencing protocols for use in small tissue samples or single cells will provide opportunities to investigate genomic changes early in the malignant process. Comparison of early- and late-stage samples (such as are being generated in TCGA) could help identify biomarkers associated with progressive disease. Whole transcriptome sequencing will likely reveal RNA splice variants and non-coding RNAs (like *PCA3*) that can be used in cancer detection (Prensner et al. 2011). Finally, very deep sequencing of cell-free DNA in the plasma or urine could be used to identify tumor-derived DNA fragments with mutations, gene fusions, DNA methylation changes, or structural rearrangements that are pathognomic for specific malignancies.

Moving forward, it is clear that progress will come only through team-based science. Work of consortia, most notably the Early Detection Research Network and the Canary Foundation, taught us that those teams must include genomic scientists, molecular imaging and laboratory medicine experts, engineers, epidemiologists, clinicians, industrial partners, and patients. Given the complexity of the carcinogenic process, cancer heterogeneity, and tumor microenvironment, it is unlikely that any single diagnostic will be effective. Rather, diagnosis will be a staged process beginning with identification of individuals at risk, performance of a targeted screen using an easily accessed biosample such as blood or urine, followed by localization of the lesions with molecular imaging (Lutz et al. 2011). Once localized, tumors can be biopsied to assess risk and select therapy. Deployment of screening technologies in the clinical setting will depend on their ability to improve clinical outcomes in an efficient and cost-effective manner.

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Translational genomics: The challenge of developing cancer biomarkers

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A model for the design and construction of a resource for the validation of prognostic prostate cancer biomarkers: the Canary Prostate Cancer Tissue Microarray

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Abstract

Tissue microarrays provide unique resources for rapid evaluation and validation of tissue biomarkers. The Canary Foundation Retrospective Prostate Tissue Microarray Resource used a rigorous statistical design, quota sampling, a variation of the case-cohort study, to select patients for inclusion in a multicenter, retrospective prostate cancer tissue microarray cohort. The study is designed to definitively validate tissue biomarkers of prostate cancer recurrence after radical prostatectomy. Tissue samples from over 1,000 participants treated for prostate cancer with radical prostatectomy between 1995 and 2004 were selected at six participating institutions in the United States and Canada. This design captured the heterogeneity of screening and clinical practices in the contemporary North American population. Standardized clinical data were collected in a centralized database. The project has been informative in several respects. The scale and complexity of assembling tissue microarrays (TMAs) with over 200 cases at each of six sites involved unanticipated levels of effort and time. Our statistical design promises to provide a model for outcome-based studies where tissue localization methods are applied to high-density tissue microarrays.

Keywords

Prostate Cancer; Prognosis; Tissue Microarray; quota sampling

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer among men in the United States, with more than 200,000 new cases expected in 2012[1]. Survival following primary treatment is generally excellent, especially among men diagnosed with presumed organ-confined disease[2]. Although approximately one-third of men undergoing surgery present with clinical factors that put them at high risk of recurrence with 10-year biochemical recurrence rates as high as 30–50%, [3][4], recent screening trials have documented that many men are diagnosed with clinically indolent disease [5,6]. These statistics suggest that there are high rates of over-diagnosis and over-treatment of prostate cancer and underlie the recent recommendation by the US Preventive Task Force against routine PSA screening of men for prostate cancer [7]. Therefore, the clinical management of prostate cancer presents patients and physicians with a paradox of localized disease that is both undertreated in some and over treated in others, highlighting the critical need to identify prognostic biomarkers of prostate cancer recurrence.

The Gleason score, clinical stage, surgical margins, lymph node involvement and pre- and post-surgery PSA values, although imperfect at predicting recurrence, are widely used in the post-operative management of patients undergoing radical prostatectomy (RP). For patients who do not elect to undergo surgery or other curative therapy, digital rectal exam (DRE), repeated biopsies and PSA levels are used to monitor disease progression [8]. Several models have been constructed to predict the probability of recurrent disease both pre- and post-operatively [9,10], with the conclusion that at most 50% of variance in outcome is explained by current prognostic parameters[11]. The predictive accuracy of these models could be improved with the addition of new prognostic biomarkers [11–13].

The identification of biomarkers that associate with prostate cancer behavior will likely be derived from a deepened understanding of the underlying biology of prostate cancer aggressiveness that includes cell proliferation, survival, invasive and migratory capabilities, angiogenesis, immune system responses, and other parameters. In addition, the application and routine deployment of biomarkers requires development and standardization of molecular tools for accurate classification of the innate biological and clinical behavior.

Once identified, new molecular biomarkers associated with high risk prostate cancer need to be tested in clinical samples with detailed follow-up and established clinical endpoints. To date, most studies have focused on developing new diagnostic biomarkers to overcome the problems with PSA testing that involves addressing poor sensitivity and specificity. As such, few resources have been available for testing prognostic biomarkers, particularly for selecting patients for immediate versus deferred treatment, and monitoring disease status over time through active surveillance. Given the challenges in developing serum-based markers of prognosis, a logical first step would be to develop biomarkers that are tissue based. Biomarker testing in tissues has been expedited by the development of tissue microarrays.

Tissue microarrays (TMAs) for identifying prostate cancer biomarkers

First described in the 1980's [14,15], tissue microarrays have been used in tissue-based studies for virtually every disease, particularly human cancer. TMAs allow simultaneous evaluation of hundreds of cases on a single histologic slide and have been used for protein and nucleotide based assay systems, most commonly immunohistochemistry and in situ hybridization. Many investigators have developed prostate cancer tissue microarrays and used them in studies designed to discover and validate candidate diagnostic and prognostic biomarkers. However, despite the identification of many candidate biomarkers, very few tissue-based biomarkers have been validated across different cohorts, and fewer have been adopted for routine clinical use. The immunohistochemical markers that are routinely used in clinical work, i.e. AMACR, p63 and ERG, have been applied exclusively for diagnostic purposes, not for prognosis. To add to the confusion, multiple studies report contradictory results for a single biomarker. For example, published reports on the family of ERG fusions have described both positive and negative associations with aggressive disease [16].

There are many reasons why prognostic biomarkers have not transitioned to routine use in the clinical management of patients with prostate cancer. Many biomarkers are presented as "candidates" based on their predicting outcome in TMAs created from whatever prostate cancer samples are on hand without a probabilistic sampling scheme from a well-defined population and, most of these studies fail to test the performance of the biomarker in the context of prognostic clinical and pathological parameters currently in use, such as Gleason patterns, clinical stage or serum PSA concentrations. Furthermore, many of these TMA patient cohorts are relatively small, with limited clinical information and short or incomplete follow-up. Even when candidate biomarkers are identified in these studies, the evaluation of the markers often stops after they are identified. Lack of validation cohorts and methods of testing for clinical significance, in addition to the somewhat mundane work of testing the many candidate biomarkers in the context of clinical and pathological parameters, likely decrease the incentive to rigorously test them as prognostic markers.

Several groups have assembled TMA cohorts with hundreds of patient samples, thereby overcoming issues of inadequate power or incomplete follow-up. However, virtually all of these cohorts are derived from surgical cases from a single institution, which may limit the generalizability of the study population with regards to patient ethnicity, disease severity, type of practice. In addition, local treatment patterns and methods of follow-up also contribute to intrinsic biases of single-institution patient cohorts. Additionally, many of these larger cohorts have significant patient heterogeneity engendered by PSA screening procedures. PSA screening has resulted in a change in the spectrum of prostate cancers in the US population, with migration over time to lower tumor stage and tumor volume. In the Prostate, Lung, Colorectal and Ovarian screening trial, Gleason grades shifted significantly to lower grades in patients detected in the first round of screening compared to those detected in subsequent rounds [17]. Many TMA cohorts include a mixture of old and

contemporary patient samples that add heterogeneity to the population but might not be relevant to current sets of patients identified by intense PSA screening.

STUDY DETAILS

Rationale and design of a multi-institutional TMA platform

The Canary Foundation Retrospective Prostate Tissue Microarray Resource (CFRPTMR) is a multicenter, retrospective prostate cancer tissue microarray study undertaken as a collaborative effort between 6 academic medical centers - Stanford University, University of California, San Francisco, University of British Columbia, University of Washington, University of Texas Health Science Center at San Antonio and Eastern Virginia Medical School. The study is supported by the Canary Foundation, Palo Alto, CA. The primary objective of the study is to *validate* biomarkers that have been reported to predict recurrent prostate cancer at the time of radical prostatectomy (RP). The secondary objective of the study is to *discover* candidate biomarkers for the prediction of non-recurrent disease. The primary study endpoints are time to recurrence and five year recurrence free survival.

The discovery and validation of clinical biomarkers in many ways parallel the steps necessary for drug development. In addition to identifying a target biomarker and developing a clinically certifiable means for measuring the biomarker, the biomarker must be tested and validated on a well-defined patient population and address a relevant clinical question. Since many tissue-based biomarker candidates have been identified and standard means of measuring the markers (e.g. immunohistochemistry) are widely used in clinical practice, we surmised that the bottlenecks to biomarker development primarily lie in validation. To address the challenges of biomarker validation, we assembled a team of pathologists, clinicians, statisticians and cancer researchers and spent two years designing and creating a tissue microarray resource for validating biomarkers of prostate cancer prognosis. As the study design emerged, it became clear that the study would follow many of the principles of a prospective clinical trial in a retrospective setting. Implementing this rigorous design involved challenges that were not anticipated in the initial study planning. Although several of the challenges were specific to prostate cancer, the resulting design features are generally applicable to most tissue-based disease studies.

We designed a common TMA platform across multiple institutions to avoid the single-institution bias. We chose to test prognostic markers in prostate tissues from a radical prostatectomy cohort. This cohort was chosen because the clinical and pathological features of the cancers could be sampled robustly (e.g. cancer grade and stage), abundant tissue was available for TMA construction, and patient outcomes were well documented. Since data suggest that some contemporary patients are over-treated [18–20], the study was designed to distinguish between indolent and aggressive disease in low and intermediate risk patients.

The clinical need to distinguish indolent from aggressive disease in men undergoing prostatectomy drove the definition of study endpoint. We selected a study outcome that captured aggressiveness and clearly defined how this outcome would be measured. The gold standard for aggressive disease is recurrent or metastatic prostate cancer. However, metastatic disease typically manifests up to 10 years after initial prostate cancer treatment [4] leading to concerns about insufficient follow-up time and spectrum bias. Prostate cancer progression after surgery is typically monitored using serum PSA concentrations as a surrogate for local recurrence or metastasis. Biochemical recurrence may identify a group of patients who are at significantly higher risk for the development of metastases and prostate cancer mortality [21]. Thus, we decided to include PSA-recurrence within 5 years of RP as a study outcome in addition to secondary/salvage therapy and clinical evidence of metastasis.

Almost all biomarker candidate studies are retrospective case-control studies and thus prone to spectrum bias in which the study sample is not representative of the clinically relevant population. For retrospective case-control studies, the cases included in the study tend to have more aggressive disease and better follow-up, both in quality of data collection and length of follow-up. Similarly, the controls included in a retrospective case-control study often represent the healthiest patients with the best follow-up. For example, metastatic prostate cancer frequently manifests 10 or more years after initial treatment for prostate cancer. The natural impulse in selecting non-recurrent patients (controls) is to limit selection to non-recurrent patients with at least 10 years of follow-up, potentially leading to spectrum bias. To help reduce this bias, a small number of censored patients (i.e. patients whose recurrent status at 5 years post-RP is unknown) are included in the study design as well as a small number of patients who experience recurrence more than 5 years after RP. To eliminate institutional selection biases, TMAs were constructed at six institutions with diverse patient populations and practice patterns. A collaboration agreement, including material transfer agreements, signed by all participating sites, allows for transfer of TMA sections among participating sites.

To accurately measure the study outcome of aggressive disease and ensure that patients met the eligibility criteria, detailed follow-up data on PSA and other clinical characteristics were required. While some sites maintained an electronic database of patient information associated with stored prostatectomy samples, others did not. These sites extracted the necessary information from medical records for each patient, a laborious and time-consuming process that shaped the sampling plan for the study. Ideally, a study cohort is drawn randomly from all eligible patients in the target population, in this case, all men undergoing prostatectomy after 1995 at the participating sites. A starting date of 1995 was selected because much of the stage shift caused by PSA screening of the US population occurred prior to that year [22].

The study used a quota-sampling plan [23](see Supplementary Materials). A random list of the entire RP cohort at each site was generated and recurrent and non-recurrent cases were identified. Participants were then chosen by moving sequentially down the list, extracting information from medical records if needed, and confirming the eligibility of each patient until the targeted number of participants in the recurrent and non-recurrent categories was obtained. This approach minimized medical records extraction since selection only continued until the target number of eligible patients was identified.

One unanticipated challenge was the time and effort required to retrieve tissue blocks that had adequate material for the TMAs after patient selection was complete. At some sites, tissue blocks for selected patients had been either consumed for other studies or were missing entirely. In some cases the growth pattern of the cancer was so serpiginous that no more than a single core could be obtained of the cancer, instead of the three cores on which the TMA design was based. After consideration of the study design and discussion, we decided that in such cases one core sufficed so that such cancers were not underrepresented in the TMAs. At several sites, substantial effort was needed to locate the missing tissues, which were often scattered in several labs where the tissues had been used for other research projects.

Patient and sample selection

The study includes tissue derived from radical prostatectomy surgical specimens. The study included samples from men with a) recurrent prostate cancer, b) non-recurrent prostate cancer and c) unknown outcome due to inadequate follow-up time (i.e. censoring). Recurrent prostate cancer is defined by 1) a single serum prostate-specific antigen (PSA) level greater than 0.2 ng/mL more than 8 weeks after RP and/or 2) receipt of salvage or

secondary therapy after RP and/or 3) clinical or radiological evidence of metastatic disease after RP. Although lower thresholds for biochemical recurrence have been proposed [24], the lower bound of sensitivity of PSA testing at some sites during the study period was limited to 0.2 ng/mL. Defining biochemical recurrence at a lower PSA value would have resulted in inconsistent application of the definition. Non-recurrent prostate cancer is defined as disease with none of the indicators of recurrence for at least five years after RP. Participants with no evidence of recurrent prostate cancer but less than five years of follow-up after RP (i.e. censored) were also eligible for the study. Inclusion and exclusion criteria and definitions of recurrent and non-recurrent disease are given in Table 1. The full study protocol is available from the authors upon request.

Data Management

The participating sites transmitted de-identified patient data for all RP patients undergoing surgery during the study period to the lead statistician in the study (ZF). The study statisticians mapped the submitted data to a set of standardized clinical variables creating a secure, centralized, database of clinical and pathological information. The statistical core checked the eligibility of each participant and returned a randomized participant list to the sites for participant selection via quota-sampling, as described below and in the Supplemental Materials.. Common data elements obtained from each institution are available on request.

Participants in the centralized database were only identified by study ID, ensuring patient confidentiality. Databases linking study IDs to patient identifying information are maintained in a locked area at each study site.

TMA Construction

The TMAs consist of formalin-fixed, paraffin embedded tissue. Each site built a set of five TMAs, in duplicate, each block containing tissue from 42 participants and 8 common control tissues (colon, tonsil, kidney, healthy prostate and liver) using an 11×16 layout (See Supplementary Material AA). For each control tissue, the tissue blocks were obtained from the same patient and distributed to the sites. Use of a common control allows for comparison of assay quality across sites.

A one mm diameter needle was used to remove tissue cores from each donor tissue block. For each case, three cores of cancer tissue were obtained from the highest grade cancer in the dominant tumor. These cancer cores generally include regions of non-neoplastic glands. In addition, one core of histologically benign prostate glandular tissue was obtained from the peripheral zone of each case altogether yielding a total of four cores per case represented on the TMA. The cores from a single participant were grouped together on the TMA. Recurrent and non-recurrent participants were randomly distributed across the TMAs. The common control tissues were grouped together providing a visual check for slide orientation.

In addition to the cores extracted for the duplicate TMAs, three cores of cancer tissue were obtained from the highest grade cancer in each case and reserved for DNA or other nucleic acid biomarker discovery or validation studies. The standard operating procedures detailing TMA construction are available from the authors on request.

TMA Distribution

A collaboration agreement, including material transfer agreements, executed at all participating sites, allows for transfer of TMA sections among participating sites. The TMA resource is also available to outside investigators. Applications to use the TMA resource are considered by the Review Committee, consisting of investigators from each participating

site. Applications are available through the Canary Foundation (www.canaryfoundation.org).

Whenever possible, digital images of stained TMA sections are uploaded and stored in a password protected web-accessible database that allows all sites to access and evaluate the images remotely. Staining is evaluated by study pathologists following standardized procedures. Evaluation procedures vary depending on the staining qualities of the particular biomarker under evaluation.

Statistical Considerations

Avoiding over-treatment of men with non-recurrent disease requires a highly specific biomarker. Hence, to validate a candidate biomarker of recurrence, we estimate the sensitivity at the threshold level associated with 98% specificity by constructing a time-dependent ROC curve for recurrence within 5 years of RP [25]. Time-dependent ROC curves offer several advantages as a tool for validating biomarkers. First, ROC curves in general are not dependent on disease prevalence. Thus, sensitivity and specificity of a biomarker can be estimated from a case-cohort study. Second, time-dependent ROC curves incorporate information from censored patients, reducing the potential bias from including only non-recurrent patients with more than 5 years of follow up.

With specificity set at 98%, we assume a biomarker must demonstrate 30% sensitivity to be clinically useful in identifying recurrent disease. This is approximately double the 15% sensitivity of Gleason score which remains the most powerful clinically applicable single variable predicting outcome in prostate cancer. The sample size needed to achieve 90% power to detect sensitivity of 30% or greater at 98% specificity is 393 recurrent patients and 393 non-recurrent patients (see Supplemental Materials for detailed calculation). The participating sites each contributed approximately equal numbers of recurrent and non-recurrent participants to the study, and the number of participants was distributed nearly evenly across the study sites. The total sample size of 1176 ensures adequate power and accounts for the 15–30% of cores that typically drop out when a TMA is sectioned [26].

Patients were selected using quota-sampling, a variation of the traditional case-cohort design described earlier. When selecting cases for study inclusion, the sampling probability for non-recurrent Gleason score 8–10 patients and recurrent Gleason 6 patients is doubled to oversample these groups of patients, who are of special interest. Table 2 details the study participant characteristics.

Project timetable

Compared to a TMA study using a convenience sample of available tissues from a single institution, construction of the Canary Foundation Retrospective Prostate Tissue Microarray Resource required a considerable increase in effort. Creating a multi-center resource coupled with a rigorous statistical design was a major effort and the time from finalizing the study protocol to construction was several years. Steps to construction included selection and standardization of clinical and pathological Common Data Elements, design and testing of the TMA layout, completion of a multi-site Material Transfer Agreement, case identification and selection of blocks based on review of sections. In particular, obtaining, reviewing, and selecting slides and blocks from potentially eligible cases required substantial effort at each site. Many slides and blocks were either not available or had been consumed by other studies. To confirm study eligibility for over 200 cases required that a pathologist at each institution review slides from at least 220 cases. This process involved annotating up to 5,500 slides (25 slides from each of 220 cases). An additional staff person was hired at several institutions to obtain slides for the pathologist to review.

DISCUSSION

The Canary Foundation Retrospective Prostate Tissue Microarray Resource is a carefully constructed TMA cohort designed to both definitively validate candidate biomarkers of aggressive disease at the time of radical prostatectomy and to discover new biomarkers for non-recurrent disease that can be used to help select patients for active surveillance. Our intent is to test these candidate biomarkers in an established a prospective, multi-institutional cohort, the Canary Prostate Active Surveillance Study (PASS), to determine whether these prognostic biomarkers can be used for selection of men at low risk for progression on active surveillance [8]. By carrying out both discovery and validation studies in tandem, we attempt to address the critical question of which patients with localized prostate cancer can be safely watched and which patients require immediate therapy.

Unlike other large prostate TMA cohorts, patients have been selected according to a strict protocol, using design features similar to a clinical trial. This design offers significant advantages in decreasing potential biases inherent in many tissue microarray studies. First, by selecting patients randomly from institutional radical prostatectomy cohorts, we minimize spectrum bias. Second, by distributing patients across institutions we make our results more generalizable by decreasing the influences engendered by local patient selection biases, differences in treatment and variations in follow-up and endpoint assessments. Third, the prospective involvement of statistical experts allowed careful definition of study endpoints and power calculations that will render positive and negative findings of tested biomarkers clinically meaningful. Our objective was to design a study in which tissue based biomarkers could be assessed using methods that were up to standards necessary for regulatory approval for use in the clinic. Given these strengths, the statistical design of this study may serve as a model for future outcomes-based studies in other diseases that employ tissue-based biomarkers. In addition, our tissue microarray is a resource available to the cancer research community for the evaluation of prognostic biomarkers with sufficient preliminary data to justify testing.

Constructing tissue microarrays using the approach and standards we have detailed entails challenges and costs. The time from initially planning to final construction and use of the microarrays was much longer than anticipated. A significant portion of that time was spent in the design of the study. However, in the long-term, we anticipate that investigators using and adapting our study design can save significant time and the output in terms of confidence in the performance of a given biomarker is enhanced. Even with our methods, study planning requires significant input from a dedicated statistician, as well as assessment of data quality from sites, and direct participation in quota sampling. In addition, use of this study design relies on the availability (or creation) of patient databases at participating institutions. These data must be transferred to the statistician(s) at a central data site in a secure and blinded fashion which requires a database manager at each site. There are also significant challenges in the construction of the TMAs. Obtaining appropriate blocks on specific selected cases from pathology archives can be rate limiting. Furthermore, as in all tissue microarray studies, our study required significant time and commitment on the part of the study pathologists, who had to review all cases, select the dominant tumor, mark the blocks for core harvesting, supervise array construction and perform quality control on the final microarrays. While these challenges can be substantial, we have demonstrated that they are surmountable.

The study design imposes certain limitations. Definitive validation of biomarkers of non-recurrent disease requires biopsy tissue taken at the time of diagnosis, i.e. when a patient would be evaluated for entry into an active surveillance program. Biopsies produce a much smaller volume of cancer for biomarker discovery and validation. This study will select a

small set of candidates for further validation with those precious biopsy samples. Other limitations include effects associated with sampling tissue blocks to construct TMAs. By using cores to represent the entire tumor, we may miss an 'index' lesion that is actually responsible for disease progression.

As we embark on assessment of prognostic biomarkers in this cohort, we will continue to test and refine the use of this resource. We anticipate that the quality of the resource will be sufficient to allow definitive testing of tissue biomarkers that they may be translated to clinical use. We encourage use of this resource by the prostate cancer research community for evaluation of mature prognostic biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Study inclusion/exclusion criteria.

Inclusion Criteria

- Radical prostatectomy surgery occurred between January 1, 1995 and September 15, 2004
- Required clinical data are available
- Required amount of tissue is available

Exclusion criteria

- PSA levels less than 0.2 ng/mL for 6 months after RP in conjunction with adjuvant therapy of any type
- Neoadjuvant hormone therapy

Recurrent prostate cancer

- Disease with evidence of recurrence at any time after radical prostatectomy as measured by:

  1) a single serum prostate-specific antigen (PSA) level greater than 0.2 ng/mL more than 8 weeks after RP

  and/or 2) receipt of salvage therapy after RP

  and/or 3) clinical or radiological evidence of metastatic disease.

Non-recurrent prostate cancer

- Disease with no evidence of recurrence for at least 5 years after RP as measured by:

  1) serum PSA level less than 0.2 ng/mL for the entire follow-up period

  and 2) no receipt of salvage therapy

  and 3) no clinical or radiological evidence of metastatic disease.

Definitions

- Neoadjuvant hormone therapy => hormone therapy of any type received prior to RP.
- Adjuvant therapy => radiation, chemotherapy or hormone treatment received less than 6 months after RP.
- Salvage therapy => radiation, chemotherapy, hormone treatment or surgery received more than 6 months after RP.

RP=radical prostatectomy

Table 2

Summary of study participants by recurrence status, follow-up time after radical prostatectomy, Gleason score and site.

	Stanford	UCSF	UW	UBC	UT	EVMS	Total
Recurrent Patients	88 (18%)	101 (20%)	102 (21%)	20 (4%)	81 (16%)	103 (21%)	495
<5 yrs. FU post-RP	72	91	92	14	71	92	432
<=6	19	25	19	1	14	48	126
3+4	27	52	27	7	24	33	170
4+3	35	10	35	3	6	6	95
8–10	11	4	11	1	25	5	57
UNK	0	0	0	2	2	0	4
>5 yrs. FU post-RP	16	10	10	6	10	11	63
<=6	2	3	3	3	6	3	20
3+4	9	5	5	1	2	5	27
4+3	4	3	1	1	1	1	11
8–10	1	0	1	1	1	2	6
	Stanford	**UCSF**	**UW**	**UBC**	**UT**	**EVMS**	**Total**
Non-recurrent Patients	84 (14%)	98 (16%)	106 (17%)	100 (16%)	127 (20%)	106 (17%)	621
<5 yrs. FU post-RP	13	9	10	19	24	12	87
<=6	1	4	4	7	15	11	42
3+4	9	4	2	11	6	1	33
4+3	2	1	1	1	2	0	7
8–10	1	0	3	0	1	0	5
>5 yrs. FU post-RP	71	89	96	81	103	94	534
<=6	22	40	52	50	55	59	278
3+4	37	41	37	22	30	32	199
4+3	9	7	6	5	6	3	36
8–10	3	1	1	4	12	0	21

RP=radical prostatectomy

FU=follow-up

Editorial

Managing Localized Prostate Cancer in the Era of Prostate-Specific Antigen Screening

James D. Brooks, MD

Despite its status as the most common cancer diagnosed in US men, with 238,590 estimated cases in 2013, prostate cancer is largely diagnosed by random blind biopsies in response to an elevated serum prostate-specific antigen (PSA) level.[1] Because most prostate cancers cannot be seen on transrectal ultrasound, the clinically and biologically most important cancers (which are usually the largest and highest grade lesions) can be missed by chance, while nonrepresentative portions of the incident cancer or secondary cancers are sampled. The uncertainty that arises from not being able to visualize cancers within the prostate has fueled 2 decades of research to improve the clinical prediction of the presence and aggressiveness of prostate cancer. In this issue on page XX, Truong and colleagues[2] present a validated tool to predict upgrading between the diagnostic biopsy and final pathology on radical prostatectomy specimens. This and other prognostic tools mark a new phase in managing localized prostate cancer and are a direct response to the current controversies in prostate cancer screening and treatment.

For most of the PSA-screening era, the primary concern has been that PSA lacks sensitivity and specificity, meaning that many men receive unnecessary biopsies for false elevations, whereas a large number of cancers are missed because of false-negative PSA levels or false-negative biopsies due to under-sampling of the prostate. The fear of missing important cancers led to several strategies to improve detection, including lowering of PSA thresholds from 4 to 2.5 ng/mL, using adjuncts such PSA velocity, and increasing the number of biopsies taken from 6 to 12 or more cores. The concerns about under-detection were amplified when an analysis of the control arm of the Prostate Cancer Prevention Trial (PCPT) showed that 15% of men had prostate cancer despite having a "normal" serum PSA level < 4 ng/mL.[3] In the context of this drive to detect every single prostate cancer, the phenomenon of upgrading was regarded more as a curiosity. Because all prostate cancer had to be found and treated, the "true" grade was intellectually interesting but of little clinical importance.

Everything changed with the reporting of the 10-year results of the Prostate, Lung, Colon and Ovarian (PLCO) and the European Randomized study of Screening for Prostate Cancer (ERSPC) trials of PSA screening.[4,5] Although PLCO demonstrated no survival benefit to PSA screening, ERSPC showed a small but significant prostate cancer-specific survival benefit at the cost of a proportionately large number of men being overtreated. These studies formed the basis for the recent US Preventive Services Task Force (USPSTF) recommendation against PSA screening for the detection of prostate cancer. One can debate the relative merits of these recommendations and these studies; however, they underscore what we have long known about prostate cancer: it occurs late in life and many men die with it rather than from it.

Although it is too early to tell what will be the effects of the USPSTF recommendations for the use of PSA screening or for prostate cancer mortality in the United States and elsewhere, it is highly unlikely that PSA screening will stop altogether. Practice patterns are slow to change generally, and patients are unlikely to forgo a screening test for a common malignancy, as has already been observed with screening mammography. Furthermore, critics of the recommendations justly point out that annual death rates from prostate cancer have dropped significantly since their peak in 1992 through 1994, and modeling attributes much of that drop to the introduction of PSA screening and subsequent increases in treatment.[6] Therefore, the next phase in management of localized prostate cancer will entail careful selection of men for treatment and expanded use of active surveillance to avoid the significant and life-altering consequences of therapy.

Active surveillance is hardly a new concept. Autopsy studies have long shown that small low-grade cancers are found commonly in men who die of other causes, with prevalence rates almost matching men's age at their time of death.[7] In

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light of that knowledge, active surveillance had long been used in T1a cancers in the era prior to PSA screening, and, as T1a cancers became increasingly uncommon after the introduction of PSA screening, has been employed in patients with small amounts of low-grade cancer (Gleason score 6 or below) on biopsy, particularly in men too old or ill to undergo therapy. Reports of very high prostate cancer-specific survival rates in relatively large cohorts of patients on active surveillance showed the safety of this approach and has added impetus to recommending this approach in low-risk patients.[8] In addition, the Göteborg site in the ERSPC trial used active surveillance frequently in low-risk patients and posted significant survival benefits in the PSA-screened arm, while avoiding treatment in 28% of men.[9] In fact, they reported needing to treat only 12 men in order to save 1 man's life, a highly acceptable ratio for any therapy.

Because of heavy PSA screening in the United States, the number of men who are candidates for active surveillance is expanding. Since the start of PSA screening, the number of men with low-stage prostate cancer has increased dramatically, whereas the number with high-stage disease has fallen.[8,10-12] Average tumor volumes at radical prostatectomy have likewise fallen,[12] and tumor volume has been associated with risk of recurrence after surgery and death from prostate cancer.[13] Screening also has led to a shift of Gleason scores to lower grades. In both PLCO and ERSPC trials, cancers discovered in the second round of screening had significantly lower Gleason scores compared with those discovered in the first round.[4,14] Interestingly, in ERSPC, much of the survival benefit was derived from first-round screening.[5] Indeed, it is likely that the shift in prostate cancer risk induced by PSA screening accounts for the lack of benefit observed in PLCO as well as the Prostate Cancer Observation Versus Observation Trial (PIVOT), which showed no overall or prostate cancer-specific survival benefit to surgery compared to observation.[15] On the other hand, the ERSPC trial and Scandinavian Prostatic Cancer Group Study Number 4 (SPCG-4) randomized trial of prostatectomy versus observation, both initiated in Scandinavia before PSA screening was widely practiced, showed benefits to active screening and treatment.[16]

In light of this shift to lower risk prostate cancer, more men should be enrolled in active surveillance. However, since 1990, the percentage of men initially managed with observation has remained stubbornly flat at approximately 9%.[17] Furthermore, in recent years, a greater proportion of men with low-risk disease are undergoing treatment with advanced technologies including intensity-modulated radiation therapy (IMRT) and robotic prostatectomy, adding to the cost of treating disease that could otherwise be managed expectantly.[11] The presumed reason for high treatment rates of low-risk prostate cancer is uncertainty of the future risk and behavior of these cancers.

If anything, the uncertainty surrounding low-risk prostate cancer is decreasing, particularly after recent modifications in Gleason grading.[18] A recent analysis of more than 14,000 radical prostatectomy cases with Gleason scores of $3 + 3 = 6$ revealed an extraordinarily low rate of concurrent lymph node metastases of 0.16% (22 cases). When the cases with lymph node metastases were regraded using contemporary criteria, all lymph node positive cases were upgraded, meaning that no case of Gleason score 6 prostate cancer had coincident lymph node metastases.[19] This finding concurs with the absence of mortality at Stanford University in Gleason score 6 cases where contemporary Gleason scoring has been practiced for many years, and is also likely true at Johns Hopkins, if one discounts Gleason score 6 cases with concurrent positive lymph nodes since they were likely misgraded.[13,20] Furthermore, the lack of association of tumor volume with recurrence or death in Gleason grade 6 cancer at the time of prostatectomy shows the indolence of these cancers.[13] Because Gleason grade 6 cancers represent nearly half of patients diagnosed currently in the United States, a large number of men could be safely managed with surveillance, were it not for the uncertainty.

As a step toward addressing this uncertainty, Truong and colleagues have developed a predictive tool for identifying patients at risk for upgrading at the time of surgery. They investigated more than 30 variables and found 4 that predicted upgrading in a multivariable model. Factors that reflect tumor volume, including number of cores involved and maximum extent of involvement on a single core, as well as PSA density have been reported previously to predict low-risk disease.[21] Somewhat surprising was the finding that obesity predicted adverse outcome. Although obesity has been linked to more aggressive cancer in some studies, others have found no association.[22] To their credit, the authors have validated this predictive tool in 2 independent datasets, with AUC (area under the curve) values above other predictive tools based on receiver operating characteristic analysis. Hopefully, this tool and others will increase physician and patient confidence that their cancer is truly low risk and thereby increase the acceptance of active surveillance.

This predictive tool is not alone in addressing the issue of upgrading and adverse prognosis. Identification

of molecular prognostic biomarkers, particularly transcripts and proteins in the tumor, has been an active area of research for years. Two tests, the Prolaris test from Myriad Genetics and the Oncotype DX prostate cancer test from Genomic Health, recently have been released for use in prostate biopsies to predict disease aggressiveness with the intent of helping select men for active surveillance.[23,24] Both tests were developed and tested in surgical cohorts and found to predict recurrence and prostate cancer mortality. The Prolaris test also has been shown to predict mortality in a watchful waiting cohort that included patients from the pre-PSA screening era and patients with a broad range of PSA values and disease states.[24] The Oncotype DX test has recently been shown to predict upgrading between the preoperative biopsy and radical prostatectomy.[23] Unfortunately, neither tool has been tested in a contemporary active surveillance cohort to see whether they predict disease progression or mortality. Although predicting mortality is a high bar, identifying patients at high risk for progression while on surveillance is an important endpoint, because earlier intervention in these men could improve their outcome. Regardless, the hope is that these tests will help men choose surveillance with greater confidence, and will add unique prognostic information beyond clinical predictive algorithms such as that of Truong et al.

Whether these or other tools will actually increase the number of men on active surveillance will depend on how they are applied. If the tools are used to identify with high confidence men who are at low risk for upgrading, the tests could actually limit the number of men placed on active surveillance. In other words, if the output of these prognostic tools is ambiguous for most men, the added uncertainty could drive them to treatment, whereas only those whose tests consistently show low risk will opt for active surveillance. In addition, it is not entirely clear whether the prediction of upgrading from Gleason score 6 to 7 represents a valid endpoint for deciding against active surveillance, even though upgrading to Gleason score 7 has been used as a progression endpoint in many active surveillance trials.[8] In the PLCO, ERSPC, PIVOT, and SPCG-4 trials, the magnitude of overtreatment of prostate cancer was so high that it had to extend into patients with Gleason scores of 7 or above. Furthermore, half of patients in the Canadian Active Surveillance trial had Gleason scores of 7, yet 8-year mortality rates were extremely low for the entire cohort (3%).[25] This raises the question whether more attention should be spent on predicting aggressiveness of Gleason score 7 cancers, in addition to accurately predicting the presence of Gleason score 6. Finally, it will be critical to investigate how these predictive tools influence the use of active surveillance and affect patient anxiety, cost of care, quality of life, and survival.

Soon, additional approaches will be deployed to help in treatment selection in men with localized prostate cancer.[26] Imaging approaches, such as multimodal magnetic resonance imaging and molecular imaging, as well as new serum and urine biomarkers, will better define which patients need to be biopsied and help refine treatment selection by improving prognostication. As each promising approach is introduced, however, constraints in health care spending will dictate that the new technology not only improve outcomes but also lower costs. Undoubtedly, these technologies that allow us to improve on PSA screening will find use in the detection of other malignancies at early stages in other organ sites. Broadly speaking, new cancer diagnostic biomarkers will be discovered for many malignancies through large consortia such as The Cancer Genome Atlas and the Early Detection Research Network and will allow us to identify malignancies early when they can be eradicated by local therapy such as surgery. However, one of the most important lessons from the past 2 decades of PSA screening and treatment is that the ability to diagnose early needs to be directly coupled with meaningful prognostication so that we do not unnecessarily overtreat lesions that are not destined to cause harm.[26]

CONFLICT OF INTEREST DISCLOSURE

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Increased Expression of *GCNT1* is Associated With Altered O-glycosylation of PSA, PAP, and MUC1 in Human Prostate Cancers

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BACKGROUND. Protein glycosylation is a common posttranslational modification and glycan structural changes have been observed in several malignancies including prostate cancer. We hypothesized that altered glycosylation could be related to differences in gene expression levels of glycoprotein synthetic enzymes between normal and malignant prostate tissues.

METHODS. We interrogated prostate cancer gene expression data for reproducible changes in expression of glycoprotein synthetic enzymes. Over-expression of *GCNT1* was validated in prostate samples using RT-PCR. ELISA was used to measure core 2 *O*-linked glycan sialyl Lewis X (sLe^x) of prostate specific antigen (PSA), Mucin1 (MUC1), and prostatic acidic phosphatase (PAP) proteins.

RESULTS. A key glycosyltransferase, *GCNT1*, was consistently over-expressed in several prostate cancer gene expression datasets. RT-PCR confirmed increased transcript levels in cancer samples compared to normal prostate tissue in fresh-frozen prostate tissue samples. ELISA using PSA, PAP, and MUC1 capture antibodies and a specific core 2 *O*-linked sLe^x detection antibody demonstrated elevation of this glycan structure in cancer compared to normal tissues for MUC1 ($P = 0.01$), PSA ($P = 0.03$) and near significant differences in PAP sLe^x levels ($P = 0.06$). MUC1, PSA and PAP protein levels alone were not significantly different between paired normal and malignant prostate samples.

CONCLUSIONS. *GCNT1* is over-expressed in prostate cancer and is associated with higher levels of core 2 *O*-sLe^x in PSA, PAP and MUC1 proteins. Alterations of *O*-linked glycosylation could be important in prostate cancer biology and could provide a new avenue for development of prostate cancer specific glycoprotein biomarkers. *Prostate 74:1059–1067, 2014.*

KEY WORDS: **prostate cancer; GCNT1; *O*-glycosylation; sialyl Lewis X**

INTRODUCTION

Most serum proteins are glycoproteins including prostate specific antigen (PSA) [1]. A growing body of evidence suggests that protein glycosylation is altered significantly in a variety of malignancies. Altered glycosylation of MUC1, for example, has been demonstrated to occur in pancreatic neoplasms and is currently being developed as a candidate early detection biomarker for testing in pancreatic cyst fluid [2–5]. Profiling using MALDI-TOF-MS or LC-MS technologies have shown significant differences between the

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branching structure of PSA glycoproteins in normal seminal plasma, with many sialylated biantennary complex oligosaccharide structures, and the prostate cancer cell line LNCaP, which contains a mixture of neutral biantennary and triantennary complex forms of oligosaccharides [6,7]. Serum PSA from patients with prostate cancer has higher levels of α2,3-linked sialic acid compared to that from seminal fluid or serum from patients with documented benign prostatic hyperplasia (BPH) [8–10]. A recent report demonstrated increased core fucosylation in prostate cancer serum glycans compared to patients with BPH [11].

Continued improvements in mass spectrometry and hybrid antibody lectin microarrays will allow unbiased detection of candidate glycoprotein biomarkers that could be useful for cancer detection in a variety of malignancies. However, limitations in sensitivity of available technologies hampers development of new biomarkers, particularly since cancer-specific glycoproteins would be expected to be found at relatively low levels in early stage malignancies, particularly when diluted in the serum of an average sized patient [12]. Since polysaccharides are synthesized by a wide array of unique enzymes, we reasoned that expression levels of those enzymes in the cancer tissue samples could provide insights into candidate glycoprotein alterations in human prostate cancer. We therefore interrogated existing prostate cancer gene expression datasets for differential expression of glycoprotein synthetic enzymes between normal and malignant prostate tissues. We identified *GCNT1* as significantly upregulated in prostate cancer. The GCNT1 glycosyltransferase catalyzes the transfer of GlcNAc from its UDP carrier to form the β1,6-linkage (core 2 branch) at GalNAc of core 1 *O*-linked glycans. To demonstrate activity by this core 2 *O*-glycan branching glycosyltransferase, we assessed the expression levels of the core 2 *O*-sLeX. This functional glycan structure is recognized by members of the selectin family of adhesion molecules [13–17], which facilitate leukocyte migration and cancer cell metastasis [18].

MATERIALS AND METHODS

Sample Collection

All prostate tissue samples used in this study were obtained from patients undergoing radical prostatectomy at Stanford University Hospital (Table I). These patients were consented for the use of their tissues under an Institutional Review Board-approved protocol. Frozen sections of these prostate samples were evaluated by a genitourinary pathologist. The tumor and adjacent normal areas were marked and contaminating tissues were trimmed away from the block as described previously [19]. Tumor samples in which at least 90% of the epithelial cells were cancerous and adjacent normal samples having no observable tumor epithelium were selected for extraction of total protein, DNA, and RNA.

TABLE I. Clinical Annotations for the Patient Samples Used

S.No.	Age	Gleason score	Stage	Pre-Op-PSA
1	74	3 + 4	pT2c	4.5
2	56	3 + 4	pT1c	18.5
3	74	3 + 4	pT3b	15.9
4	69	3 + 3	pT2c	3.4
5	69	4 + 5	pT1c	10.3
6	58	3 + 3	pT2c	12
7	56	3 + 3	pT2c	4.1
8	51	3 + 4	pT2a	12.7
9	56	4 + 3	pT3b	38.48
10	72	3 + 4	pT3a	5.3

Reagents

Monoclonal antibodies against PSA, PAP, and MUC1 were purchased from Meridian (Saco, ME), Cosmo Bio Co (Tokyo, Japan) and Abnova (Taipei City, Taiwan) respectively. For detection of the core 2 *O*-linked glycan sialyl Lewis X (sLe^x), we used the CHO131-monoclonal antibody which has been characterized previously [17]. Tissue lysis buffer and Halt Protease Inhibitor Cocktail were purchased from Thermo Scientific (Rockford, IL). Antibody coating buffer was purchased from Immunochemistry Tech (Bloomington, MN). The PSA standard used in this study was prepared previously in the Department of Urology at Stanford University [20]. The PAP standard and MUC1 recombinant protein were purchased from Mybiosource (San Diego, CA) and Abnova (Taipei City, Taiwan) respectively. HRP-streptavidin and peroxidase substrates were purchased from R&D (Minneapolis, MN).

Gene Expression Datasets and Analysis

Gene expression data was analyzed using a dataset containing 81 prostate cancer samples and 52 normal prostate tissue samples harvested from radical prostatectomy specimens [19]. Transcript levels were normalized across the microarrays and extracted for the following glycosylation genes: O-Linked Glycosylation: *A4GNT*, *B3GALTL*, *B3GNT8*, *B4GALT5*, *C1GALT1*, *C1GALT1C1*, *GALNT1*, *GALNT10*, *GALNT11*, *GALNT12*, *GALNT13*, *GALNT14*, *GALNT2*, *GALNT3*, *GALNT4*, *GALNT6*, *GALNT7*, *GALNT8*, *GALNT9*, *GALNTL1*,

GALNTL5, *GALNTL6*, *GCNT1*, *GCNT3*, *GCNT4*, *OGT*, *POFUT1*, *POFUT2*, *POMGNT1*, *POMT1*, *POMT2*, *ST3GAL1*, *ST3GAL2*, *ST6GALNAC1*, *ST8SIA3*, *ST8SIA6*. N-Linked Glycosylation: *AGA*, *B3GNT2*, *B3GNT3*, *B3GNT8*, *B4GALT1*, *B4GALT2*, *B4GALT3*, *EDEM1*, *EDEM2*, *EDEM3*, *FUCA1*, *FUCA2*, *FUT11*, *FUT8*, *GANAB*, *GLB1*, *GNPTAB*, *GNPTG*, *HEXA*, *HEXB*, *MAN1A1*, *MAN1A2*, *MAN1B1*, *MAN1C1*, *MAN2A1*, *MAN2A2*, *MAN2B1*, *MANBA*, *MGAT1*, *MGAT2*, *MGAT3*, *MGAT4A*, *MGAT4B*, *MGAT4C*, *MGAT5*, *MGAT5B*, *MOGS*, *NAGPA*, *NEU1*, *NEU2*, *NEU3*, *NEU4*, *PRKCSH*, *ST6GAL1*, *ST8SIA2*, *ST8SIA3*, *ST8SIA4*, *ST8SIA6*, *UGGT1*, *UGGT2*. Relative expression levels between the prostate cancer and normal tissues were compared using the two-class significance analysis of microarray (SAM) test [21].

For the datasets from LaPointe et al. [22] and Gulzar et al. [19], data from each microarray were mean centered across the array prior to data extraction. To look at relative levels of expression between adjacent normal and malignant tissues, expression levels were mean centered across experiments. For experiments from Stamey et al. [23], Singh et al. [24], Taylor et al. [25], and Glinsky et al. [26] and Sboner et al. [27], raw expression levels were normalized to the median array intensity. For *GCNT1*, absolute expression levels were plotted and expression levels compared using the Student's *t*-test.

TaqMan Gene Expression Assay

RNA expression levels of *GCNT1* were measured in four benign adjacent and 15 prostate tumor samples using the TaqMan Gene Expression Assay. We used Applied Biosystems inventoried assays with FAM/MGD labeled probes (Hs01922706_s1) and the Human *B2M* (beta-2-microglobulin) as an endogenous control. Total RNA (1 μg) was reversed transcribed to cDNA and assayed in triplicate using the Stratagene Mx3005P QPCR System in accord with manufacturer's protocols. Using MxPro qPCR software, the average CT and delta-CT were calculated for *GCNT1* and normalized between samples by integrating the average CT value from the *B2M* to obtain the delta-delta-CT.

Protein Extraction and ELISA Assay

Prostate tissues (approximately 100 mg each) were homogenized in 400 μl of lysis buffer containing protease inhibitors at 4°C for 2 min. The tissue lysates were centrifuged at 10,000 × g in 4°C for 5 min and supernatants containing total proteins were collected, aliquoted and stored at −80°C until use. Antibodies (100 μl) against PSA (M66276M, Meridian), PAP (Hyb-7412 Cosmo Bio) and MUC1 (H00004582-AP41, Abnova) were immobilized on ELISA plates at 4°C overnight. The ELISA plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) and then blocked with 1% BSA in PBS buffer, pH 7.4 for 90 min. Total protein extract from paired normal and prostate tumor tissues were added to the appropriate wells and incubated at room temperature for 60 min. The plates were washed three times with PBST followed by addition of 100 μl (2 μg/ml) biotinylated anti-sLe^x detection antibody. In parallel experiments, total PSA, PAP, and MUC1 proteins were also measured with 100 μl (0.5 μg/ml) detection antibodies against PSA (Meridian *M86506M-biotin*), PAP (Cosmo Bio *Hyb-7432-biotin*), and MUC1 (Abnova H00004582-AP41). These proteins were detected by streptavidin-HRP based detection method according to manufacturer's instructions (R&D Biosystems-USA). The levels of sLe^x glycoprotein signals were normalized to tissue PSA, PAP, and MUC1 protein levels. The normalized data for normal and malignant tissues was compared using a paired *t*-test.

RESULTS

We interrogated a gene expression dataset of 81 tumors and 52 adjacent normal prostate tissue samples harvested at radical prostatectomy for transcript levels of 84 glycosylation linked enzymes [19]. We used a two class SAM to assess for differences in levels of gene expression between normal and tumor samples. Of the 84 transcripts, 25 were significantly up-regulated and 7 were significantly down-regulated. However, only 7 of these transcripts showed expression differences of 1.5-fold or more: *GCNT1* (2.5-fold), *GALNT7* (2.1-fold), *ST6GALNAC1* (1.8-fold), *GALNT3* (1.7-fold), *B4GALT3* (1.6-fold), *MOGS* (1.6-fold), and *EDEM3* (1.5-fold). Notably, *GCNT1*, *GALNT7*, *GALNT3*, and *GALNT1*, all of which catalyze synthesis of *O*-linked glycoproteins, were expressed at significantly higher levels in malignant compared to normal prostate tissue samples ($P < 0.001$) (Fig. 1A). Of these transcripts, *GCNT1* was the most differentially expressed between normal and malignant prostate tissues and we focused our analysis on this transcript. GCNT1 catalyzes the transfer of GlcNAc from its UDP carrier to form the β1,6-linkage to the α-GalNAc of core 1 *O*-linked glycan that is bound to Ser/Thr residues of polypeptides to form the *O*-glycan core 2 branch (Fig. 1B).

We validated the gene expression data with quantitative PCR for *GCNT1* on a set of normal and malignant prostate cancer samples. In agreement with the microarray data, *GCNT1* transcript levels were increased in the cancer samples compared to those in normal prostate tissues (Fig. 2). Together these data strongly suggest that *GCNT1* is over-

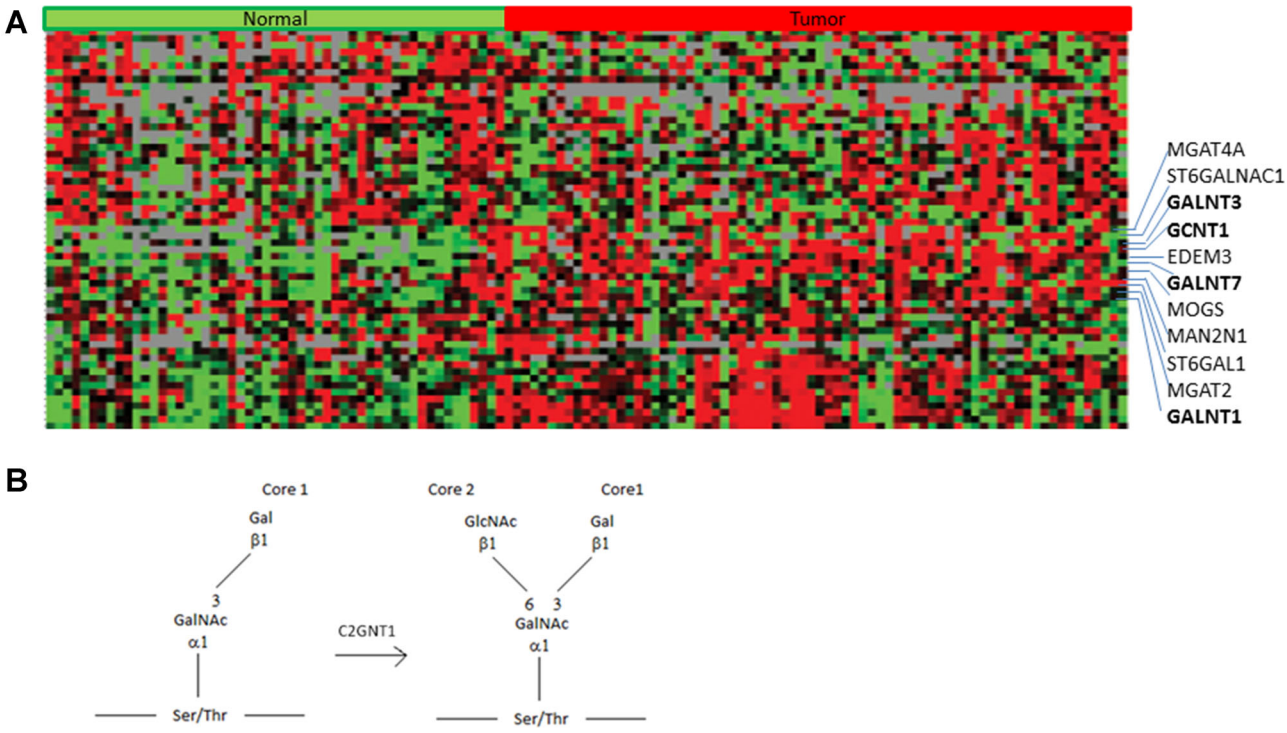

Fig. 1. **A**: Two-class SAM analysis comparing 56 benign adjacent normal and 81 prostate cancer samples. Listed are transcripts differentially expressed between the two groups with a False Detection Rate (FDR) < 0.05. Each tumor sample is represented in a column and individual transcripts are displayed in rows. Red indicates relative increased expression level of transcripts relative to the median level across the samples, whereas green represents relative decrease in expression levels, and the degree of color saturation corresponds to the degree of change. **B**: *GCNT1* gene product β-1,6-N-acetylglucosaminyltransferase-1 (C2GNT1) catalyzes formation of the core 2 branch in O-linked glycans.

expressed in prostate cancer compared to normal prostate tissues.

We further investigated expression levels of *GCNT1* in other publicly available datasets that had data from normal and malignant prostate tissues. *GCNT1* transcript levels were significantly elevated ($P < 0.001$) in malignant tissues compared to normal prostate tissues datasets from LaPointe et al. [22] and Singh et al. [24] (Fig. 3). To better understand how altered levels of *GCNT1* varied across the spectrum of normal prostate tissue, prostate dysplasia, low grade cancer and high grade cancer, we interrogated gene expression levels in a third independent set of carefully dissected prostate tissues samples generated by Stamey et al. [23]. *GCNT1* levels were higher in prostate cancer samples and dysplasia compared to benign prostatic hyperplasia (Fig. 4). *GCNT1* transcript levels in cancers with pure Gleason pattern 3 did not differ significantly from those with pure Gleason pattern 4 (Fig. 4). However, there appeared to be a slight trend toward higher expression in pattern 4 compared to pattern 3 cancer as has been reported previously [28].

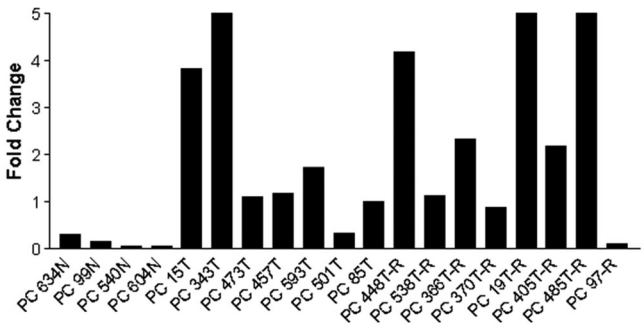

Fig. 2. Normalized *GCNT1* expression levels obtained by TaqMan RT-PCR gene expression assay (N, normal; T, Tumor; R, biochemical recurrence following surgery).

Given this trend, we assessed whether *GCNT1* transcript levels predicted clinical outcome in available gene expression datasets with associated clinical follow-up. *CGNT1* expression levels appeared normally distributed across tumor samples, therefore samples were divided at the median into high and low expressing tumors. Tumors expressing higher *GCNT1* transcript levels were associated with a significantly increased risk of biochemical recurrence in the Glinsky et al. [26] dataset ($P < 0.001$, Log rank test) while *GCNT1* gene expression levels were not associated with biochemical recurrence in data from Gulzar

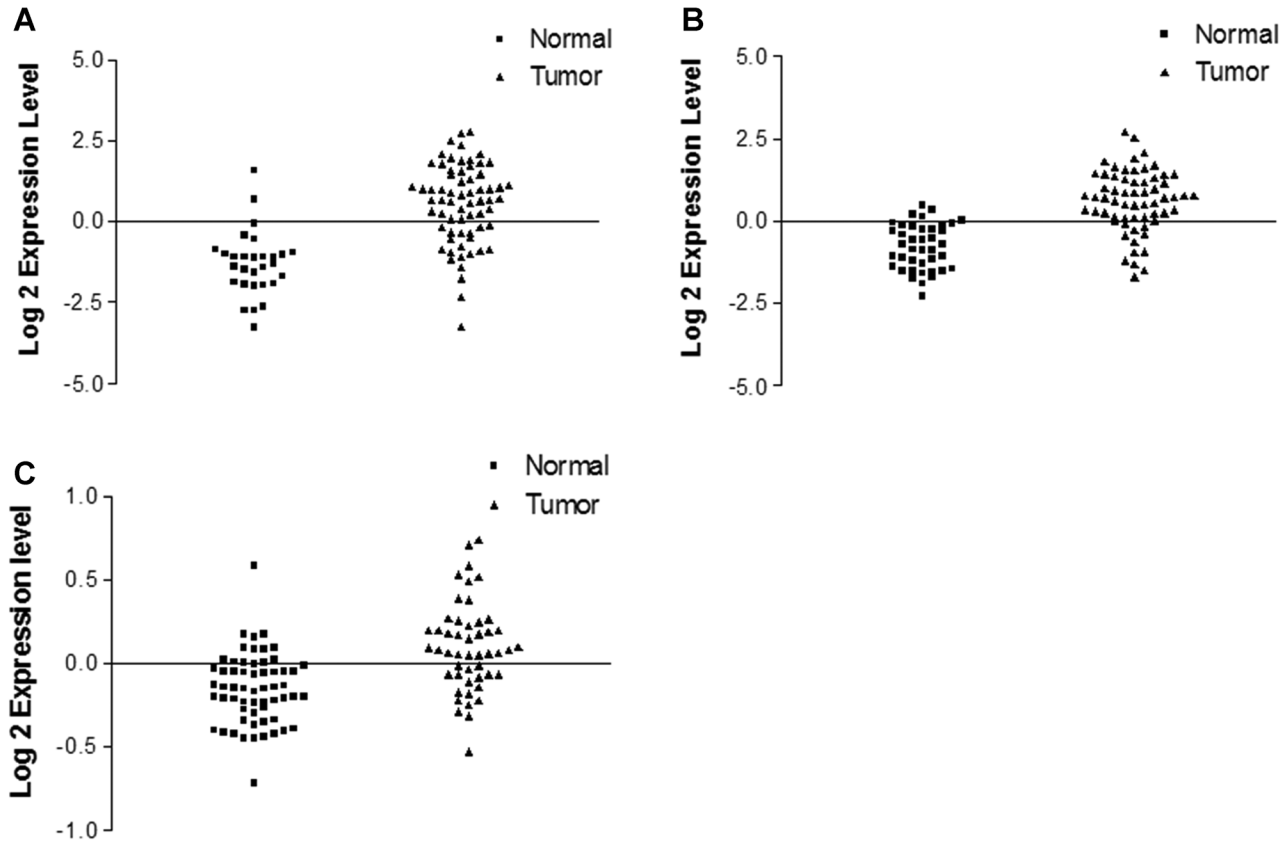

Fig. 3. Transcript levels of *GCNT1* in normal and malignant tissues from human prostate tissue gene expression datasets: A) Gulzar et al. (2012), B) LaPointe et al. (2004), C) Singh et al. (2001). Mean-centered normalized log2 fluorescence ratios for *GCNT1* from normal and prostate cancer samples are shown.

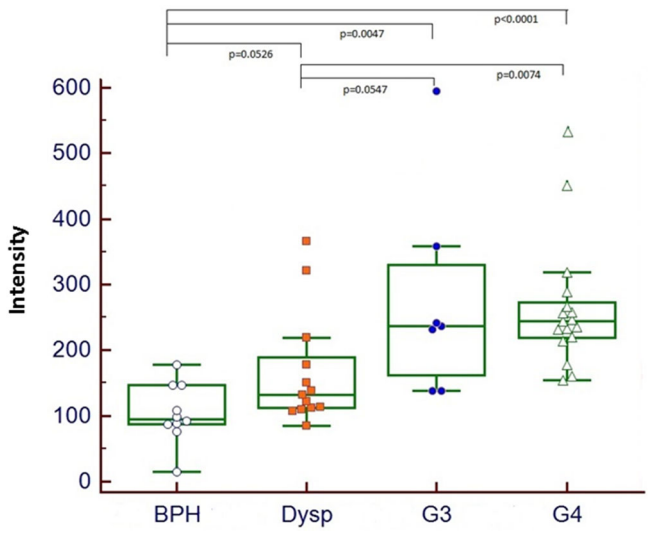

Fig. 4. Comparison of *GCNT1* transcripts levels in microdissected tissues from human BPH (n = 10), prostatic dysplasia (n = 13), pure Gleason pattern 3 prostate cancer (n = 7) and pure Gleason pattern 4/5 prostate cancer (n = 17). Normalized log2 intensity ratios from the Affymetrix U133A gene chip set are shown.

et al. [19] ($P = 0.11$) and Taylor et al. [25] ($P = 0.22$), nor were expression levels associated with prostate cancer death in Sboner et al. [27] ($P = 0.08$). Although *GCNT1* transcript levels did not reach significance, it is notable that lower *GCNT1* transcript levels trended with increased post-surgical biochemical recurrence in the Gulzar dataset [19] and with prostate cancer specific mortality in the Sboner dataset [27]. This is in contrast to the Glinsky dataset and previous reports [28], where higher transcript levels were associated with biochemical recurrence and potentially calls into question the association between *GCNT1* levels and prostate cancer aggressiveness.

Since GCNT1 and other *O*-glycoprotein synthetic enzymes were significantly over-expressed in cancer, we next examined the glycosyltransferase's functional activity to determine whether this occurred at higher levels in malignant compared to normal prostate tissues. We selected three proteins to interrogate for glycosylation changes in prostate tissues: two proteins specifically expressed in the prostate: prostate specific antigen (PSA) and prostatic acid phosphatase (PAP),

as well as Mucin1 (MUC1), a glycoprotein we have identified as over-expressed in aggressive prostate cancers and with documented glycosylation changes observed in other cancer types [2–5,22].

Total protein was extracted from 10 paired normal and malignant prostate tissue samples harvested fresh from radical prostatectomy specimens (Table I). We developed sandwich ELISA immunoassays for total PSA, PAP, and MUC1 protein levels, and a parallel assay to measure levels of the *O*-glycan core 2 branch sLe^x structure on PSA, PAP and MUC1 using a detection antibody (CHO-131) specific for the capping structure sLe^x on core 2 branched *O*-glycans. CHO-131 does not recognize the structurally similar sLe^a glycan branch structure [17] Total PSA, PAP, and MUC1 protein levels did not differ significantly between normal and malignant prostate tissues ($P = 0.14$, $P = 0.15$, $P = 0.86$, respectively) (Fig. 5A–C). However, levels of core 2 *O*-linked sLe^x modified PSA and MUC1 (normalized by total PSA or MUC1 levels in each sample) were significantly higher in the cancer tissues compared to the paired normal prostate tissues (Fig. 5D and E; PSA $P = 0.03$; MUC1 $P = 0.01$). Similarly, total PAP levels did not differ between normal and malignant prostate tissues, while normalized PAP core 2 *O*-sLe^x levels were found at higher levels in the cancer tissues compared to the paired normal prostate tissues that approached statistical significance ($P = 0.06$) (Fig. 5F).

DISCUSSION

By interrogating a set of transcripts encoding for glycoprotein synthetic enzymes, we were able to take advantage of large existing prostate cancer gene expression data sets to identify *GCNT1*, as well as other enzymes involved in O-linked glycosylation, as being transcriptionally up-regulated in malignant compared to normal tissues. This finding was consistent across several datasets encompassing several hundred specimens, showing that up-regulation of *GCNT1* is a common feature in prostate cancer. Increased expression occurred in pre-cancerous dysplasia, low grade and high-grade prostate cancer, implying it is an early change in prostate carcinogenesis. In addition, using sandwich ELISA assays, we were able to document increased *O*-linked glycosylation with sialyl Lewis X in PSA, MUC1, and PAP proteins in malignant prostate tissues compared to adjacent normal prostate tissues. The finding of altered *O*-linked glycosylation in all three proteins investigated suggests that this change could be pervasive across many glycoproteins in prostate cancer.

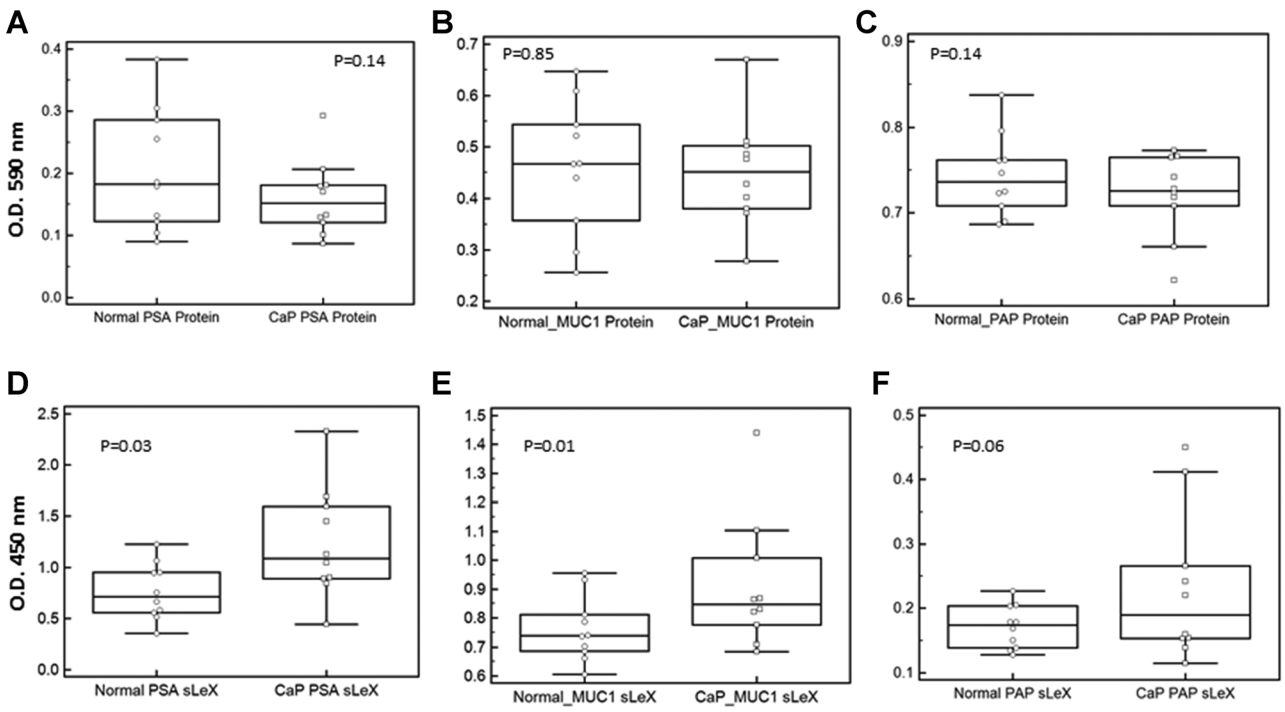

Fig. 5. Total PSA, MUCI, and PAP protein levels and parallel measurements of core 2 *O*-sLe^x modified PSA, MUCI, and PAP glycoprotein levels. Upper panels show total protein values (A590): **A**: PSA, (**B**) MUCI and (**C**) PAP. All were normalized to total protein levels in each sample. Bottom panels: core 2 *O*-sLex values (A450/A590) normalized to cognate protein levels: **D**: PSA core 2 *O*-sLex, **E**: MUCI core 2 *O*-sLex and (**F**) PAP core 2 *O*-sLex.

Altered protein glycosylation has been studied in target proteins in prostate cancer previously, although most work has centered on *N*-linked glycosylation changes. PSA is one of the best-studied proteins and has been used as a standard for comparison of different methods of assessing *N*-linked glycoprotein structure [29]. PSA derived from cancer appears to have a greater number of *N*-linked branch structures compared to PSA from benign prostate tissues [6,7] However, far less has been reported regarding *O*-linked glycoproteins in prostate cancer. GCNT1 protein levels assessed by immunohistochemistry have been reported previously to be up-regulated in 45 of 69 prostate cancer cases, and higher expression was associated with more aggressive disease [28]. Stable expression of GCNT1 in LNCaP cells resulted in larger tumor growth in an orthotopic mouse model as well as increased adherence of the cells to collagen IV, although GCNT1 expression did not alter growth kinetics in vitro [28]. We did not find consistent association of *GCNT1* transcript levels with clinical outcome in four large datasets, although it is possible that protein levels do not correlate directly with transcript levels. Regardless, the finding of increased GCNT1 protein expression in human prostate cancers agrees with our finding of up-regulation of transcript levels of *GCNT1* and other *O*-linked core 2 synthetic enzymes, as well as our finding of increased core 2 *O*-sLe^x in PSA, MUC1, and PAP in malignant compared to normal prostate tissues. Furthermore, our findings are consistent with previous reports of increased levels of α2-3-linked sialic acid on serum glycoproteins in patients with prostate cancer compared to men with BPH [11].

Cancer-associated alterations of *O*-linked glycosylation, particularly of core 2 *O*-sLe^x, have been observed in a variety of contexts and could influence prostate carcinogenesis through several different mechanisms. For example, core 2 *O*-linked glycosylation of MUC1 on the surface of prostate cancer cells allows them to evade immune destruction by NK cells [30]. Similarly immune cloaking has been observed in other cancer types and core 2 *O*-linked glycosylation of MUC1 also has been shown to block antigen presentation to CD8+ T cells [31]. Aberrant *O*-linked glycosylation of the extracellular domain of MUC1 also has been shown to mediate up regulation of β-catenin and ERK1/2 cell signaling pathways through the MUC1 cytoplasmic tail region [32,33]). In addition, *O*-glycosylation of oncofetal fibronectin has been reported to drive the epithelial to mesenchymal transition (EMT) induced by transforming growth factor-β (TGF-β) in prostate cancer cells [34], and abundant evidence implicates the TGF-β signaling pathway in prostate cancer genesis and progression [35]. Furthermore, a growing body of evidence suggests that core 2 *O*-sLe^x expression on the cell surface of circulating tumor cells (CTCs) can facilitate the interaction of circulating tumor cells and endothelial selectins and integrins, which could facilitate CTC tethering, rolling motion and eventual extravasation of cancer cells [26,36]. Since we observed core 2 *O*-sLe^x modifications on all three proteins we tested, it is plausible that other proteins in prostate cancer cells show similar changes, meaning that alterations in *O*-glycosylation could act through several mechanisms to facilitate prostate cancer cell growth.

Altered *O*-glycosylation is being explored broadly in cancer detection and treatment, and could be applied to prostate cancer management. For example, several platforms have been developed using lectins or core 2 *O*-sLe^x specific antibodies to detect *O*-glycosylation changes in tissues, serum and plasma samples of patients in pancreatic and colon neoplasms that could be developed as detection biomarkers [37]. In pancreatic cancer, knock-down of the *O*-glycosylation synthetic enzyme GalNAc-T3 has been shown to attenuate growth and induce apoptosis, suggesting that this enzyme activity could be targeted therapeutically [38]. In addition, *O*-linked glycosylation changes in cancers have been reported to be the targets of immunotherapy and to influence immune activity [31]. Since we have found evidence for pervasive changes in *O*-glycosylation in prostate cancer, it is possible that these strategies could be used to improve specificity of prostate cancer detection or provide new therapeutic avenues including improved immunotherapy.

CONCLUSIONS

By leveraging gene expression data in human prostate samples, we have identified increased expression of *GCNT1* and other *O*-glycoprotein synthetic enzymes as a relatively common event in prostate cancer. In addition, we document that these changes in expression appear to have functional consequences based on our finding of increased levels of core 2 *O*-sLe^x modifications in PSA, MUC1 and PAP in human prostate cancers compared to paired normal prostate tissues. *O*-linked glycosylation changes might be found in additional proteins in prostate cancer and should be explored for the biological effects and therapeutic potential, and as a possible source of cancer-specific biomarkers.

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The Prostate 75:1206–1215 (2015)

A Multicenter Study Shows *PTEN* Deletion Is Strongly Associated With Seminal Vesicle Involvement and Extracapsular Extension in Localized Prostate Cancer

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BACKGROUND. Loss of the phosphatase and tensin homolog (*PTEN*) tumor suppressor gene is a promising marker of aggressive prostate cancer. Active surveillance and watchful waiting are increasingly recommended to patients with small tumors felt to be low risk, highlighting the difficulties of Gleason scoring in this setting. There is an urgent need for predictive biomarkers that can be rapidly deployed to aid in clinical decision-making. Our objectives were to assess the incidence and ability of *PTEN* alterations to predict aggressive disease in a multicenter study.

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METHODS. We used recently developed probes optimized for sensitivity and specificity in a four-color FISH deletion assay to study the Canary Retrospective multicenter Prostate Cancer Tissue Microarray (TMA). This TMA was constructed specifically for biomarker validation from radical prostatectomy specimens, and is accompanied by detailed clinical information with long-term follow-up.

RESULTS. In 612 prostate cancers, the overall rate of *PTEN* deletion was 112 (18.3%). Hemizygous *PTEN* losses were present in 55/612 (9.0%) of cancers, whereas homozygous *PTEN* deletion was observed in 57/612 (9.3%) of tumors. Significant associations were found between *PTEN* status and pathologic stage ($P < 0.0001$), seminal vesicle invasion ($P = 0.0008$), extracapsular extension ($P < 0.0001$), and Gleason score ($P = 0.0002$). In logistic regression analysis of clinical and pathological variables, *PTEN* deletion was significantly associated with extracapsular extension, seminal vesicle involvement, and higher Gleason score. In the 406 patients in which clinical information was available, *PTEN* homozygous ($P = 0.009$) deletion was associated with worse post-operative recurrence-free survival (number of events = 189), pre-operative prostate specific antigen (PSA) ($P < 0.001$), and pathologic stage ($P = 0.03$).

CONCLUSION. *PTEN* status assessed by FISH is an independent predictor for recurrence-free survival in multivariate models, as were seminal vesicle invasion, extracapsular extension, and Gleason score, and preoperative PSA. Furthermore, these data demonstrate that the assay can be readily introduced at first diagnosis in a cost effective manner analogous to the use of FISH for analysis of *HER2/neu* status in breast cancer. Combined with published research beginning 17 years ago, both the data and tools now exist to implement a *PTEN* assay in the clinic. *Prostate 75:1206–1215, 2015.*

KEY WORDS: **active surveillance; Gleason score; biomarker; PI3K/PTEN/Akt pathway; fluorescence in situ hybridization; tissue array analysis**

INTRODUCTION

Screening, detection, and treatment of prostate cancer remain highly controversial [1,2] and PSA screening has led to the potential over-diagnosis and over treatment of low-risk disease [3,4]. Radical prostatectomy and radiation therapy are treatments that offer high cure rates; however, it is estimated that over 1,055 men need to be screened and 37 cancers detected to prevent one prostate cancer death 11 years later [5]. Recently, recommendations for screening have been narrowed [6], and active surveillance protocols are commonly recommended for men with low risk disease [7,8]. Prognostic biomarkers are needed to aid in clinical decision making for these men to more accurately distinguish between low risk and moderate to high risk prostate cancer.

Currently, the clinical tools available for making these treatment decisions include PSA level, number of positive core biopsies, percent of cores involved by tumor, and Gleason score. Various nomograms are used to facilitate clinical decision making using these data [9]. However, the Gleason score of the biopsy sample, which remains the most powerful prognostic marker, is inaccurate in a large percentage of patients especially when only a small volume tumor is sampled during biopsy. The vast majority of biopsies are scored as either Gleason 6 or 7 and yet up grading or down grading occurs in 14–51% and 9%, respectively when comparing the Gleason score of the biopsy to that found in the prostatectomy specimen [10–12]. Likewise, clinical stage poorly estimates final pathological stage [13], an important predictor of clinical outcome, second only to Gleason score [14]. There is a need for biomarkers that distinguish aggressive from indolent forms of prostate cancer. It is difficult to address the utility of prognostic markers for prostate cancer in a formal prospective study. While randomized studies remain the gold standard for diagnostic and therapeutic trials, several constraints preclude this. Follow-up times of 8–10 years are required to prospectively assess clinical outcomes and the need to evaluate promising biomarkers in a reasonable time frame drives translational studies of prostate cancer toward retrospective analysis of prostatectomy specimens. Active surveillance and watchful waiting protocols have focused attention on the difficulties in grading small tumors. Thus the widely used tools for risk assessment for active surveillance urgently require additional informative biomarkers to supplement Gleason scores [15]. As such, there is an absolute necessity to rigorously evaluate all emerging biomarkers to improve pre-treatment assessment of Gleason score and pathological stage so that urologists and patients can make well-informed treatment decisions at first diagnosis.

Prostate cancer biomarker assays must perform well not only in prostatectomy specimens, but must

also be effective when only small amounts of tissue are available, as is the case in core needle biopsies at the time of initial diagnosis. Tissue microarrays (TMAs) place small samples of many cases on a single slide for rapid evaluation and validation of tissue biomarkers.

The phosphatase and tensin homolog (*PTEN*) is a tumor suppressor gene that can be deleted in patients with prostate cancer [16,17]. *PTEN* was initially studied in human prostate tumors using molecular techniques such as microsatellite analysis [18]. Molecular methods are not readily adaptable to the clinical laboratory, and immunohistochemistry (IHC) is a useful and cost-effective tool for biomarker analysis. IHC studies of PTEN protein were long hampered by the lack of a robust antibody [19]. Fluorescence in situ hybridization (FISH) has therefore been frequently used, and genomic deletions of *PTEN* have been reported in 20–30% of prostate carcinomas [20–22], and are associated with aggressive disease [23,24]. These well-annotated studies have indicated that loss of the *PTEN* gene independently predicts more aggressive disease and poorer outcomes in prostate cancer. However, virtually all of these cohorts were derived from surgical cases from a single institution, which may limit the generalizability of the study population with regards to patient ethnicity, disease severity, and type of practice. In addition, local treatment patterns and methods of follow-up also contribute to intrinsic biases of single-institution patient cohorts. The Canary Foundation Retrospective Prostate Tissue Microarray Resource [25] includes samples from 1,116 subjects treated for prostate cancer with radical prostatectomy between 1995 and 2004 from six participating institutions in the United States and Canada. These samples were ideal to evaluate the role of PTEN as a biomarker to help identify aggressive prostate cancer for implementation to supplement existing predictive tools. Using *PTEN* FISH probes optimized for sensitivity and specificity [26], our objectives were to confirm the ability of *PTEN* deletions to predict aggressive disease, and to determine an expected incidence of PTEN loss in a multicenter study. The accumulated clinical data, combined with newly available probes for FISH and new reagents for IHC published by others [19] open the door to implementation of PTEN assays in the clinical setting.

MATERIALS AND METHODS

Tissue Specimens and TMA Design

The Canary Foundation Retrospective Prostate Tissue Microarray Resource [25] is a retrospective prostate cancer TMA built with the collaboration of six academic medical centers: Stanford University, University of California, San Francisco, University of British Columbia, University of Washington, University of Texas Health Science Center at San Antonio, and Eastern Virginia Medical School. The TMAs contained cores from 1,116 patients undergoing radical prostatectomy between 1995 and 2004. For each case, three cores of cancer tissue were obtained from the highest grade cancer in the dominant tumor. In addition, one core of histologically benign prostate glandular tissue was obtained from the peripheral zone of each case yielding a total of four cores per case on the TMA. The TMA was constructed to assess biomarkers that provide prognostic information independent of clinical and pathological information. The AJCC pathologic staging system was used [27] with stages pT1 and pT2 being combined, as were stages pT3 and pT4. For practical purposes, the vast majority of cases were stages pT2 and pT3. The cases included samples from men with biochemically recurrent prostate cancer within 5 years of surgery and non-recurrent prostate cancer after 5 years of follow-up. In addition, non-recurrent cases censored prior to 5 years and with recurrence after 5 years were included to correct for spectrum bias [25]. Recurrent prostate cancer is defined by one of the following: A single serum PSA level >0.2 ng/ml more than 8 weeks after radical prostatectomy; salvage or secondary therapy after radical prostatectomy; clinical or radiologic evidence of metastatic disease after radical prostatectomy. Although lower thresholds for biochemical recurrence have been proposed [28], the lower bound of sensitivity for PSA testing at some sites during the study period was limited to 0.2 ng/ml. Non-recurrent prostate cancer was defined as disease with none of the indicators of recurrence for at least 5 years after radical prostatectomy. There was no central pathology review in this cohort. The prostatectomy specimens were therefore scored prior to the modification introduced by the International Society of Urological Pathology (ISUP) [29]. We oversampled recurrent cases of Gleason score $3+3=6$ and $3+4=7$ as well as non-recurrent cases with Gleason score $4+4=8$. While this strategy diminishes the prognostic significance of Gleason score, it improves power to discover biomarkers that provide prognostic information independent of Gleason score [25].

Fluorescence In situ Hybridization

The *PTEN* Del TECT FISH utilizes a four-color probe combination as described [26]. Probes were supplied by CymoGenDx LLC (New Windsor, NY) as follows: centromeric copy control probe-CYMO-Red;

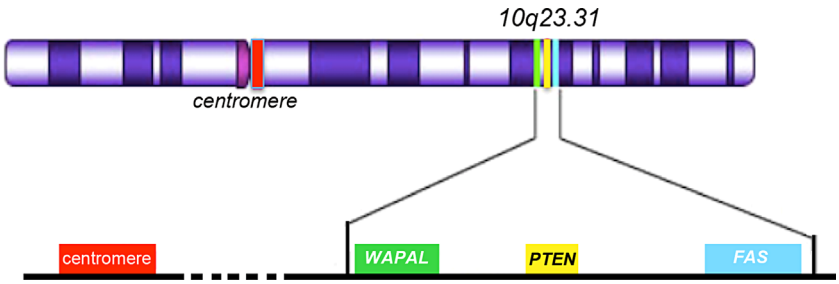


Fig. 1. Schematic diagram of chromosome 10 showing genomic locations and respective positions of the four-color FISH probe used. The relative probe length and color are shown on the linear map at the bottom ofthefigure by the length of the rectangle.

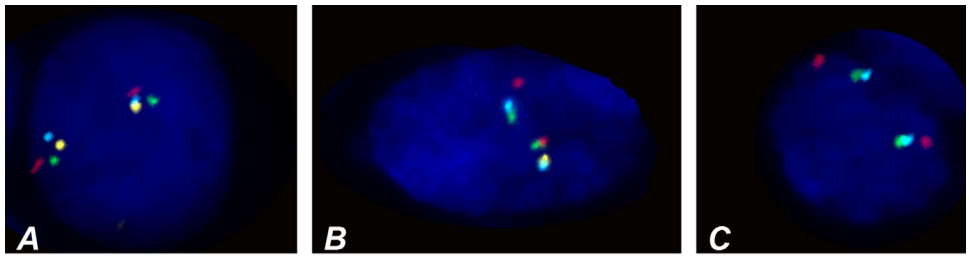
WAPAL – CYMO-Green; *PTEN* – CYMO-Orange; and *FAS* – CYMO-Aqua (Fig. 1). The two probes *WAPAL* and *FAS* on either side of *PTEN* provide information about the size of larger deletions and also allow recognition of artifactual losses of *PTEN* due to histologic sectioning. Artifacts in assessing *PTEN* loss can arise when histologic sectioning cuts away the *PTEN* locus in cells in the section while leaving the centromere in place. The latter is a result of the long distance between the centromere and the *PTEN* locus on chromosome 10. Loss of all three probes distal to the centromere in a small fraction of cells was regarded as artifact, whereas consistent loss of a single copy of *PTEN* in >50% of cells was scored hemizygous deletion. We have shown previously that use of the probes bracketing *PTEN* improves the fidelity of assessments of *PTEN* loss [26]. FISH analysis was performed using 5 μm TMA sections stained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) in areas selected by the pathologist using an immediately adjacent section stained with hematoxylin and eosin. *PTEN* copy number was evaluated by counting spots for all four probes using SemRock filters appropriate for the excitation and emission spectra of each dye in 50–100 non-overlapping, intact, interphase nuclei per tumor TMA core. For each case, two cores were scored based on the overall quality of FISH hybridization. In cases where different clonal deletions were present, all three cores were analyzed. Hemizygous (single copy) *PTEN* loss was assigned when >50% of nuclei exhibited clonal loss of *PTEN* and adjacent probes. Homozygous deletion was defined by a simultaneous lack of both *PTEN* locus signals in 30% of scored nuclei [30].

Statistical Methodologies

Summary statistics of PTEN deletion status and other patient characteristics were provided in frequencies and percentages. Fisher's exact test was used to assess correlation between PTEN deletion status and other characteristics. Pre-surgery PSA was summarized using mean, standard deviation, and range. Comparison between PTEN deletion status groups with respect to pre-surgery PSA was performed using the Kruskal–Wallis test. A logistic regression model was used to assess correlation between PTEN deletion status and other clinical factors. A Cox model was used to assess the effects of PTEN status (homozygous deletion, hemizygous deletion, vs. undeleted for PTEN), and other patient characteristics of recurrence-free survival (RFS), where an event was defined as clinical recurrence, salvage treatment, metastasis, or death due to prostate cancer. A backward elimination procedure was used to identify final multivariate models. Factors of interest may be forced into the final model to account for their effects even if not significant. All tests were two-sided and *P*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC).

Study Population

PTEN gene status was studied in 3,150 cancer cores derived from 1,116 cases and controls. FISH results were obtained from 641 cases encompassing 1,160 tissue cores in total. Of the 409 cases excluded for technical reasons, 70 were due to inadequate tumor tissue, and 339 could not be analyzed because poor tissue digestion prevented adequate hybridization. There was no apparent bias in the distribution of technical failures across the six different study sites in the cohort. Tissue cores can hybridize with variable efficiencies on a TMA slide due to differences in aging and fixation effects from the tissue in the donor blocks. Unfortunately, it was not possible to optimize pretreatment digestion times for all cores as only one TMA slide was available for FISH. This meant that the proportion of successfully hybridized cores (61%) was lower than is usually reported. Of the 641 cases successfully studied by FISH, 29 cases were excluded from further analysis because the clinical information was inadequate resulting in 612 analytical cases.



D

PTEN status	Signal configuration (Centromere; WAPAL PTEN FAS)
PTEN Intact by FISH (two copies)	
Hemizygous PTEN deletion (one copy)	
Homozygous PTEN deletion (no copies)	

Fig. 2. **A**: Representative signal pattern observed when the PTEN gene is intact and two copies ofthe gene and allchromosome 10 probes are present as two copies. **B**: Nuclear signal pattern observed for PTEN hemizygous deletions. **C**: Homozygous PTEN deletion (both copies lost). **D**: Scoring schemaused to classify FISH signals present in interphase nuclei based on the colored labels used for each probe. The schema only shows examples with simple interstitial deletions affecting the PTEN gene (yellow spot loss) only. In some tumors, larger deletions extending from WAPAL (green) to FAS (blue) were detected. In addition, five tumors with loss arising as a monsomy of chromosome 10 were detected.

PTEN Deletion Analysis

Homozygous or heterozygous *PTEN* deletion was seen in 112 (18.3%) of 612 tumor samples. Hemizygous *PTEN* deletion accounted for 55 of the 612 (9.0%) adenocarcinomas, whilst homozygous *PTEN* deletion was found in 57 of the 612 (9.3%) tumors. The counting scheme and representative images of undeleted, hemizygous, and homozygous deletions are shown in Figure 2. Of the 55 tumors with a homozygous deletion, 16 had interstitial deletions involving *PTEN* alone, with both flanking genes (*WAPAL* and *FAS*) being retained. The remaining 39 homozygous losses had larger deletions on one chromosome, with the majority extending in a telomeric direction, so that the *FAS* gene was more commonly deleted than *WAPAL*. The distribution of deletion size in the hemizygously deleted tumors was similar to the homozygous deletions, but 4/57 tumors had *PTEN* loss as part of a monosomy 10.

PTEN Deletion Correlated With Gleason Score

PTEN deletion status correlated very strongly with increasing Gleason score ($P = 0.0002$) (Table I). Furthermore, *PTEN* status (undeleted, hemizygous deletion, and homozygous deletion) showed a step-wise correlation with Gleason score. Undeleted *PTEN* was more commonly observed in Gleason score 6 tumors, while homozygous deletion was more common in Gleason score 8 cancers. For example, homozygous deletions were found in 18% (11/62) Gleason score >8 cancers, while only 3% (8/243) of Gleason <6 cases had a homozygous *PTEN* deletion. The Gleason 7 tumors fell between these extremes with 12% (26/225) of 3+4 tumors having a homozygous deletion and 16% (12/76) of 4+3 having homozygous deletions. For the hemizygous *PTEN* deletions, there was no apparent relationship between Gleason score and presence of a *PTEN* loss.

TABLE I. Association of *PTEN* Deletion Status With Clinical Parameters of Progression

	PTEN deletion status								
	Undeleted		Hemi-deletion		Homo-deletion		All		
	N	%	N	%	N	%	N	%	*P*-value*
Margin									
Missing	100	20.00	5	9.09	12	21.05	117	19.12	
Positive	156	31.20	19	34.55	14	24.56	189	30.88	0.60
Negative	244	48.80	31	56.36	31	54.39	306	50.00	
Pathology stage									
Missing	115	23.00	12	21.82	8	14.04	135	22.06	
pT1/pT2	284	56.80	26	47.27	21	36.84	331	54.08	<0.0001
pT3/pT4	101	20.20	17	30.91	28	49.12	146	23.86	
Seminal vesicle invasion									
Missing	9	1.80	0	0	0	0	9	1.47	
No	468	93.60	49	89.09	47	82.46	564	92.16	0.0008
Yes	23	4.60	6	10.91	10	17.54	39	6.37	
Extra-capsular invasion									
Missing	6	1.20	0	0	0	0	6	0.98	
No	380	76.00	38	69.09	28	49.12	446	72.88	<0.0001
Yes	114	22.80	17	30.91	29	50.88	160	26.14	
Gleason score									
Missing	5	1.00	1	1.82	0	0	6	0.98	
≤6	216	43.20	19	34.55	8	14.04	243	39.71	0.0002
3 + 4	178	35.60	21	38.18	26	45.61	225	36.76	
4 + 3	58	11.60	6	10.91	12	21.05	76	12.42	
≥8	43	8.60	8	14.55	11	19.30	62	10.13	
Total	500	100.00	55	100.00	57	100.00	612	100.00	

*Fisher's exact test.

Association of *PTEN* Deletion With Parameters of Aggressive Disease

PTEN deletion was significantly associated with higher pathologic stage, presence of seminal vesicle invasion, extracapsular extension, and increased Gleason score (Table I). Each of these adverse pathological findings increased in cases with homozygous deletion compared to hemizygous deletion, suggesting a gene dosage effect. However, there was no association between surgical margin involvement and the presence of *PTEN* deletion. A multivariate Cox proportional hazard model was used to assess whether *PTEN* status, and clinical and pathological variables predicted survival after radical prostatectomy. A backward elimination procedure was used to identify significant factors in the final model. In 406 evaluable patients, there were 189 events and *PTEN* homozygous deletion was strongly associated with post-operative RFS (homozygous $P = 0.009$, HR 1.64) as were pre-operative PSA and seminal vesicle invasion ($P < 0.0001$ for both; Table II). Neither *PTEN* status nor the clinicopathological variables correlated with the development of metastatic disease or prostate cancer death; however, there were only 30 events among the 612 evaluable patients (data not shown).

PTEN Deletion Correlated With Pathology Stage, Extracapsular Extension, and Seminal Vesicle Invasion

PTEN deletion status showed a highly significant correlation with pathologic stage ($P < 0.0001$). For the stage pT3/pT4 tumors, 19% (28/146) had homozygous deletions compared to only 6% (21/331) of stage pT1/pT2 tumors. This effect was less pronounced for the hemizygous deletions with 12% (17/146) stage pT3/pT4 tumors showing deletions and 8% (26/331) of stage pT1/pT2. Both extracapsular extension (pT3a) and seminal vesicle invasion (pT3b) are associated with a high risk of recurrence after radical prostatectomy. The presence of a *PTEN* deletion correlated strongly with seminal vesicle invasion ($P = 0.0008$), with homozygous deletion having the strongest predictive value. Seminal vesicle invasion was present in 18% (10/57) of tumors with a

homozygous deletion compared to only 5% (23/491) of tumors without a *PTEN* deletion. Similarly, extracapsular extension correlated strongly with *PTEN* deletion ($P < 0.0001$). Extracapsular extension was present in 51% (29/57) of tumors with homozygous *PTEN* deletion, compared to only 23% (114/494) of tumors without *PTEN* deletion. For the 55 tumors with a hemizygous *PTEN* deletion, this effect was still present although less marked, with 6/55 (11%) having seminal vesicle invasion and 17/55 (31%) with extracapsular extension. A logistic regression model confirmed that tumors with homozygous *PTEN* deletions had a significantly higher probability of having extracapsular extension, seminal vesicle invasion, and higher Gleason scores compared to undeleted *PTEN* (Table III). However, for these clinical features, tumors with hemizygous deletions did not show significant difference in comparison to tumors without a *PTEN* deletion.

DISCUSSION

The clinical dilemma facing urologists is how to treat newly diagnosed low and intermediate risk prostate cancer. Treatment options include active surveillance, prostatectomy, hormonal therapy, and radiation therapy. Consequently, there is an intensive search for biomarkers to help distinguish the more aggressive from less aggressive tumors. The search for useful biomarkers in the blood has been slow and difficult [31] and over detection of indolent disease remains a significant problem for prostate cancer [32]. A sample of tissue itself, therefore, remains a mainstay to determine the prognosis of disease. Existing methods for measuring biomarkers in tissue include RNA expression arrays [33], DNA analysis, immunohistochemistry (IHC), and fluorescence in situ hybridization (FISH). An advantage of FISH is that it can be applied to small amounts of tumor in 18 gauge biopsies and fits well into existing work flows, consuming minimal amounts of tissue.

The *PTEN* gene and protein have shown promise in identifying aggressive prostate cancer. Loss of PTEN protein function is strongly associated with key properties of the aggressive cancer phenotype such as cell survival, proliferation, migration, adhesion, and invasion [34]. Our findings agree with other studies showing that prostate cancers with *PTEN* gene deletions have shorter recurrence free survival [35,36]. Moreover, homozygous *PTEN* deletion in tumors is strongly associated with castrate resistant disease, metastasis [23], and prostate cancer specific death [20–22]. Both *PTEN* deletions and the presence of the prostate cancer specific gene fusion, most notably *TMPRSS2:ERG*, have been associated with worse outcome in prostate cancer patients. However, the role of *TMPRSS2:ERG* fusions as a primary determinant of prognosis remains unclear [37–39]. The use of TMAs has many advantages in evaluating the performance of a biomarker in large studies such as this. While IHC is typically used to evaluate biomarker expression in TMAs, we successfully queried TMAs constructed at multiple sites using FISH. In the past, there have been concerns that *PTEN* FISH could be deployed in prostate core biopsies bearing small amounts of tumor if it cannot be successfully utilized in TMAs which similarly have small amounts of tissue.

While this study and the cumulative results of previous PTEN studies [20–24] are not strictly comparable to prospectively obtained biopsies, a thoughtfully designed retrospective study of prostatectomy specimens may offer more accurate results than a prospective study of prostate biopsies. Examination of prostatectomies remains the most accurate assessment of the pathologic stage of prostate cancer. Studies based solely on clinical staging remain hampered by lack of accurate pathologic staging. In the near future, improved imaging methods may fill this gap, but for the time being, pathologic staging based on prostatectomy remains a gold standard for staging. Furthermore, the centerpiece of risk assessment for prostate cancer remains Gleason score. Upgrading at radical

TABLE II. Multivariate Cox Proportional Hazard Model for Recurrence-Free Survival (RFS)

Factor	Comparison	Hazard ratio	95% hazard ratio confidence limits		Pairwise *P*-value	Overall *P*-value
Pre-op PSA	1 unit increase	1.04	1.02	1.05	<0.0001	
PTEN	Homo vs. no deletion	1.64	1.13	2.37	0.009	0.02
	Hemi vs. no deletion	1.28	0.84	1.95	0.25	
Seminal vesicle invasion	Yes. vs. No	2.31	1.53	3.48	<0.0001	
Gleason score	3 + 4 vs. ≤6	1.54	1.12	2.11	0.008	<0.0001
	4 + 3 vs. ≤6	2.34	1.59	3.45	<0.0001	
	≥8 vs. ≤6	2.31	1.52	3.53	<0.0001	

TABLE III. Logistic Regression Model Correlating PTEN With ECE, SV, and Gleason Score

Endpoint	Parameter	Comparison	Odds ratio	95% LCL	95% UCL	Pairwise *P*-value	Overall *P*-value
Extra-capsular invasion (yes, no)	PTEN	Homo vs. no del	3.45	1.97	6.06	<0.0001	<0.0001
		Hemi vs. no del	1.49	0.79	2.70	0.20	
	PTEN	Any del vs. no del	2.32	1.51	3.57	<0.0001	
Seminal vesicle invasion (yes, no)	PTEN	Homo vs. no del	4.33	1.87	9.43	0.0003	0.002
		Hemi vs. no del	2.49	0.89	6.07	0.06	
	PTEN	Any del vs. no del	3.39	1.70	6.62	0.0004	
Gleason ($\leq$6, 7, $\geq$8)	PTEN	Homo vs. no del	3.37	1.83	6.19	<0.0001	<0.0001
		Hemi vs. no del	1.54	0.82	2.89	0.23	
	PTEN	Any del vs. no del	2.32	1.55	3.48	<0.0001	

prostatectomy still occurs in 26–50% of prostatectomies as compared to biopsy Gleason score [40]. Thus, challenges in grading of biopsies include interobserver variability, particularly in assessing small tumors in biopsies, and the upgrading which occurs when prostatectomy specimens are examined.

Improved antibodies have led to the use of IHC in the evaluation of PTEN expression as a surrogate for *PTEN* deletions and point mutations, and IHC fits the work-flow of diagnostic pathology and can be readily deployed at modest cost [13]. However, interphase FISH analyses can be performed on less than 100 tumor cells, so it is now feasible to obtain clinically useful genetic information such as *PTEN* status by applying FISH to tumor tissue in needle core biopsies. There is timeliness, therefore, in assessing the applicability of *PTEN* FISH analysis to prostate cancer risk assessment at first diagnosis. Sampling issues and heterogeneity of PTEN loss within a tumor may diminish the negative predictive value of PTEN assays [41]. However, the loss of PTEN has independent predictive value, and can be assayed alone or in combination with other emerging biomarkers such as TMPRSS-ERG that may add additional information. Using HER-2 status in breast cancer as a model [42], the availability of both IHC and FISH assays for PTEN status offers the prospect of implementing a widely studied biomarker. Two recent studies using prostate cancer needle biopsies [19,43] have shown a strong association between PTEN loss, as determined by immunohistochemistry, and poor outcome. Collectively, these recent data using needle core biopsies and the findings of this present manuscript draw attention to the value of PTEN as a predictive biomarker for intermediate risk prostate cancer and suggest a possible clinical workflow for assessing PTEN status. For example, initial analyses of PTEN expression could be carried out using immunohistochemistry with established methods [13,19]. Regions of tumor or suspicious areas in the biopsy that are PTEN weak or otherwise indeterminate by IHC could then readily be studied by FISH using the refined probes we describe.

PTEN deletion is a clinically meaningful finding, and we suggest that a combination of IHC and FISH can detect PTEN deletions if they are present in the sampled tissue. The upgrading that frequently occurs from biopsy to prostatectomy exemplifies the limitations of current clinical decision making tools deployed at the time of biopsy. Our analysis of small core samples of tumor tissue on the TMA suggest that clinically meaningful assessments of PTEN status and prognosis can be made in the context of biopsies. This finding, coupled with studies demonstrating the prognostic value of PTEN expression by IHC on biopsies suggests that PTEN testing of biopsy samples could be a useful adjunct for patients with low and intermediate risk prostate cancer in making therapeutic decisions. However, additional studies will be necessary to determine the best use of PTEN IHC, PTEN FISH, or a step-wise assessment of both in patients newly diagnosed with low and intermediate risk prostate cancer who are deciding between treatment and active surveillance. Furthermore, the false negative rate for PTEN status, particularly in the biopsy setting, has not been determined definitively and will require additional study.

Proper cancer staging is also critical in clinical decision-making in early stage prostate cancer. For example, it is well known that extracapsular extension or seminal vesicle invasion increases the risk of recurrence [44,45]. The prostate biopsy rarely gives information about extracapsular extension or seminal vesicle invasion because the sample rarely includes

those areas for evaluation. Our study demonstrates an association with *PTEN* loss, particularly in cases with homozygous deletion, and extracapsular extension, and seminal vesicle invasion. Therefore, the association of *PTEN* loss with pathological up-staging, and the consequential increased risk provide additional information that could be useful in clinical decision making.

This large multicenter retrospective TMA analysis of radical prostatectomy specimens shows that homozygous *PTEN* deletion is associated with higher stage, higher Gleason score, and a higher incidence of both extraprostatic extension, and seminal vesicle invasion. In addition, homozygous deletion of *PTEN* is associated with shorter RFS in men after radical prostatectomy. Our findings, suggest that *PTEN* deletion testing of biopsies could provide an important additional tool to assist urologists and patients making treatment decisions when faced with low and intermediate risk prostate cancer. Given the strong associations loss of *PTEN* by FISH and IHC, future studies will be needed to define optimal workflows using these methods to best define prognosis.

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PLOS ONE

RESEARCH ARTICLE

Evaluation of ERG and SPINK1 by Immunohistochemical Staining and Clinicopathological Outcomes in a Multi-Institutional Radical Prostatectomy Cohort of 1067 Patients

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Abstract

Distinguishing between patients with early stage, screen detected prostate cancer who must be treated from those that can be safely watched has become a major issue in prostate cancer care. Identification of molecular subtypes of prostate cancer has opened the opportunity for testing whether biomarkers that characterize these subtypes can be used as biomarkers of prognosis. Two established molecular subtypes are identified by high expression of the ERG oncoprotein, due to structural DNA alterations that encode for fusion transcripts in approximately ½ of prostate cancers, and over-expression of SPINK1, which is purportedly found only in ERG-negative tumors. We used a multi-institutional prostate cancer tissue microarray constructed from radical prostatectomy samples with associated detailed clinical data and with rigorous selection of recurrent and non-recurrent cases to test the prognostic value of immunohistochemistry staining results for the ERG and SPINK1 proteins. In univariate analysis, ERG positive cases (419/1067; 39%) were associated with lower patient age, pre-operative serum PSA levels, lower Gleason scores ($\leq$3+4=7) and

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improved recurrence free survival (RFS). On multivariate analysis, ERG status was not correlated with RFS, disease specific survival (DSS) or overall survival (OS). High-level SPINK1 protein expression (33/1067 cases; 3%) was associated with improved RFS on univariate and multivariate Cox regression analysis. Over-expression of either protein was not associated with clinical outcome. While expression of ERG and SPINK1 proteins was inversely correlated, it was not mutually exclusive since 3 (0.28%) cases showed high expression of both. While ERG and SPINK1 appear to identify discrete molecular subtypes of prostate cancer, only high expression of SPINK1 was associated with improved clinical outcome. However, by themselves, neither ERG nor SPINK1 appear to be useful biomarkers for prognostication of early stage prostate cancer.

Introduction

Based on high incident rates of 230,000 cases per year, significant mortality rates of 29,000 men yearly, and a relatively slow natural history, prostate cancer should be an ideal target for screening interventions to impact survival [1]. The drop in death rates from 40,000 cases per year to current rates suggests that PSA screening has made an impact on prostate cancer mortality [2]. However, results from prospective randomized screening and surgical intervention trials, particularly the Prostate Lung, Colon and Ovarian (PLCO) and PIVOT trials in North America, have raised questions as to the effectiveness of screening to decrease deaths [3, 4]. While the ERSPC trials and SPCG-4 conducted in less heavily screened populations of Europe showed benefits to PSA screening and surgical treatment for prostate cancer specific mortality [5, 6], taken together all of the trials highlight potential over-screening and over-treatment of prostate cancer as major risks, particularly in light of the morbidities associated with prostate cancer treatments [7].

Much as therapies targeted to discrete molecular lesions are making an impact in the management of advanced cancers, the concept of using molecular markers to identify aggressive and potentially lethal cancers has gained traction in managing early stage prostate cancer [8]. Evidence from the intervention trials as well as observations of the high prevalence of prostate cancer at autopsy suggest that there is a very large pool of prostate cancers that should not be diagnosed and do not require therapy [9]. Current clinical markers, including tumor stage, serum PSA levels and biopsy Gleason score, lack sufficient predictive power across all clinical scenarios to confidently select patients who do not harbor future risk of disease progression and can be safely observed; therefore, identification of molecular features that correlate with aggressive disease is a high priority.

To address the need for validation of candidate biomarkers of disease aggressiveness, we have developed a prostate cancer tissue microarray (Canary prostate TMA). The TMA resource was constructed at 6 participating centers using a common protocol of radical prostatectomy specimens with complete clinical data and long-term follow-up [10]. These TMAs had a rigorous statistical design including random case selection, case sampling schemes to minimize spectrum biases, and oversampling of cases in specific groups of interest to help in identifying biomarkers that best predict failure after radical prostatectomy, a surrogate for aggressive disease.

Prostate cancers are characterized by over-expression of the ETS transcription factor ERG as a result of a somatically acquired fusion event to the regulatory region of the TMPRSS2 gene [11]. These gene fusions are found in nearly half of prostate cancers and are thought to

constitute a distinct molecular subtype of the disease. Over-expression of SPINK1 has been described in cancers lacking the TMPRSS2-ERG fusion and has been reported to identify a subset (approximately 5–10%) of prostate cancers that behave more aggressively [12]. Conflicting results have been reported on whether ERG and SPINK1 over-expression is associated with adverse outcome (summarized in [13] and [14]). We tested whether either biomarker, whether alone or in combination, predicted outcomes after radical prostatectomy in our multi-institutional TMA resource.

Materials and Methods

Ethics Statement

Tissue blocks and accompanying clinical data were collected at each of the participating sites (Stanford University, University of California San Francisco, University of Washington, University of British Columbia, University of Texas Health Sciences Center at San Antonio, Eastern Virginia Medical School) under a research protocol developed by the investigators with IRB approval at each institution. The approved protocols included sharing of de-identified data and samples and correlation of clinical data with biomarker data acquired from the TMAs. A materials transfer agreement was developed jointly and approved at each site for sharing of tissue microarrays and tissue samples.

TMA cases and construction

For case selection, de-identified clinical data were submitted to the statistical core (lead statistician ZF) for random case selection. Constraints were placed on selection such that recurrent cases in patients with Gleason score 3+3 = 6 and non-recurrent cases in those with Gleason score 4+4 = 8 were oversampled. In addition, cases were selected to attempt to balance the number of recurrent and non-recurrent cases at each site. Details of case selection, tissue microarray construction and statistical considerations have been detailed elsewhere [10].

Once cases were selected, tissue blocks were obtained at each site. In cases where tissue blocks were not available, additional cases were selected in accord with a random list generated by the data repository. Tissue microarrays were constructed at each participating site in accord with a standard protocol. Briefly, 3 cores of the highest grade cancer from the largest cancer area were harvested as 1 mm cores and transferred to the recipient block. In addition, one core of histologically normal prostate tissue was included from each case. Once constructed, the TMAs were baked and stored under nitrogen gas at each site.

Immunohistochemistry (IHC)

Freshly cut 5 micron sections from each site were shipped to Stanford University for immunohistochemical staining. ERG immunohistochemistry was performed using a commercial rabbit monoclonal antibody to ERG (clone EPR3864; 1:100; Epitomics, Burlingame, CA, USA) as described previously [15]. SPINK1 expression was assessed with a mouse monoclonal antibody (1:50 dilution; H00006690-M01, Abnova) [14]. In addition, TMAs were stained with hematoxylin and eosin (H & E) as well as immunohistochemical staining using a mouse monoclonal antibody (34bE12, Dako) for high molecular weight keratins (HMWK). The H&E and HMWK slides were scanned to digital images using a Leica SL801 autoloader and SCN400 scanning system (Leica Microsystems; Concord, Ontario, Canada) at magnification equivalent to ×20 and images of individual cores were viewed and scored using the SlidePath digital imaging hub (DIH; Leica Microsystems) of the Vancouver Prostate Centre and share online with Canary pathology team. Scoring was performed on-line for the presence of cancer in each core on the

TMA, and only cases with cancer were scored for ERG and SPINK1 (all performed by a single pathologist: JKM).

TMAs from one institution had technically insufficient staining for ERG and were, therefore, excluded from the analysis, leaving a total of 1067 patients who were included in this analysis. For SPINK1, the percentage of neoplastic cells demonstrating cytoplasmic staining were recorded for each individual core based on distinct expression patterns that were recognized: 0- no staining, 1- less than 50% of cells staining in scattered individual cells, 2- less than 50% of cells staining in complete glands, 3–50–80% of cells staining, 4- greater than 80% of cells staining. The SPINK1 staining score 4 was based on identical criteria utilized by Tomlins et al. as an independent predictor of biochemical recurrence [12]. For ERG, the staining was scored for each individual core as follows: 0- no staining, 1- faint nuclear staining visualized at high power magnification, 2- strong nuclear reactivity easily seen at low power magnification (100X magnification or less). The criteria utilized for an ERG score 2 were identical to those that have been shown to correlate with fusion status [15, 16]. For each antibody, the highest score recorded for a case in any of its three individual cores was utilized in the statistical analysis for that individual patient.

Statistical methods

The primary endpoint of this analysis was post-surgery recurrence-free survival (RFS) where the baseline was set at the date of surgery. RFS was defined as absence of PSA (biochemical) recurrence, local recurrence, prostate cancer metastases, or death from prostate cancer, with events scored at the earliest date noted after surgery. Disease-specific survival (DSS), defined as death from prostate cancer or development of advanced metastatic disease, and overall survival (OS) were secondary endpoints. SPINK1 and ERG score for each patient was the maximum score of all the cores from that patient as defined above.

Summary statistics of patients' SPINK1, ERG, and combined staining status were provided in frequencies and percentages. Fisher's exact test was used to assess the association between ERG and SPINK1 status with each other and with patient characteristics. Kaplan-Meier (KM) method was used to estimate survival endpoints by patient group. Cox proportional hazard model was used to estimate effects of ERG and SPINK1 on each survival endpoint. Unweighted and weighted analyses were performed, with the latter accounting for the oversampling of patients with recurrence less than 5 years after surgery. All tests were two-sided and p-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC). Kaplan Meier plots were generated using Spotfire S+8.2 (TIBCO Inc., Palo Alto, CA). The complete dataset of clinical, pathological and staining data can be found in S1 File.

Results

Patient population

After exclusion of TMAs from 1 study site for technical issues, a total of 1067 patients had evaluable ERG or SPINK1 status by IHC. The mean age of the entire cohort was 61.7 ± 7.2 (range 35 to 80) and mean PSA was 8.7 ± 8.8. For ERG, a total of 113 cases (11%) did not have evaluable staining data either because of core loss or because lack of cancer in the core samples. Of the remaining tumors, 44% (419/954) showed strong ERG expression (score 3), 53% (506/954) showed no expression (score 0), with the remaining showing faint ERG expression (score 1) (29/954 or 3%) (Fig 1A).

For SPINK1, immunostaining results were available on 90% (963/1067) of cases with 104 cases lacking interpretable staining data. SPINK1 expression was strongly positive (score 4) in

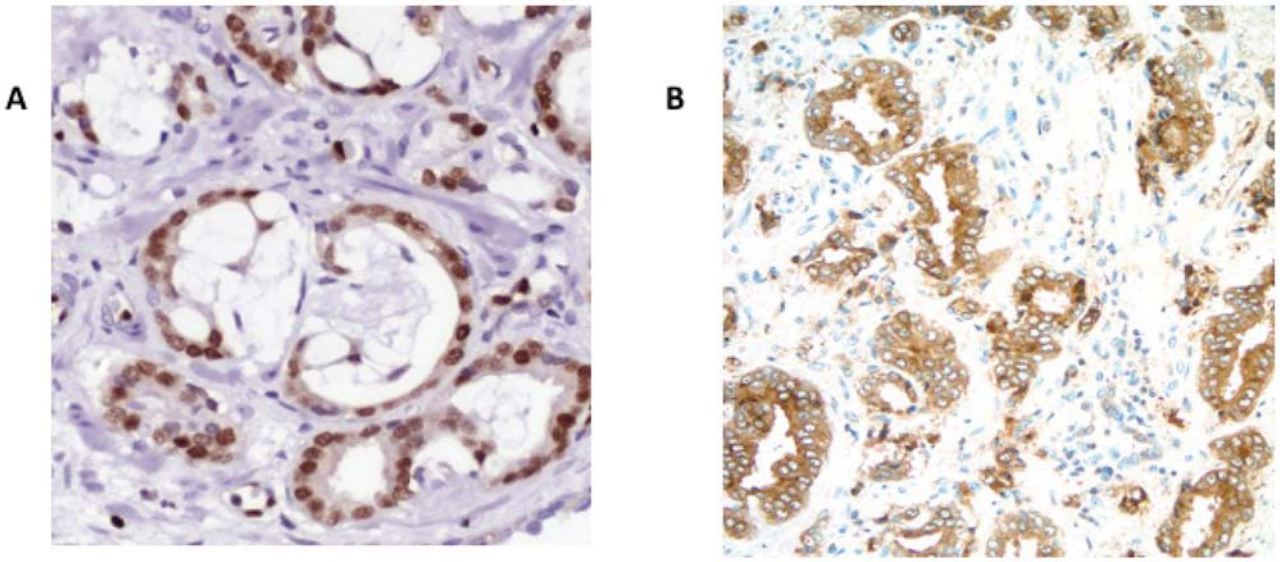

Fig 1. Immunohistochemical staining showing high level expression of A) ERG – nuclear staining, and B) SPINK1 with cytoplasmic staining.

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3.4% of cases (33/963) and absent from 86% (826/963) with the remaining 104 (11%) cases showing varying degrees of faint staining (Fig 1B). Of 954 patients with evaluable SPINK1 and ERG staining, 3 cases had strong expression of both SPINK1 and ERG protein, although this overlap was lower than expected by chance ($P<0.0001$, Fisher's exact test). Staining results and clinical data are summarized in Table 1.

ERG /SPINK1 expression and clinicopathological variables

High-level expression of ERG (score 2) and SPINK1 (score 4) by IHC were tested for their association with clinical and pathologic features (Table 2). Neither ERG nor SPINK1 expression was associated with pathological findings of seminal vesicle invasion, positive surgical margins or extracapsular extension. ERG positive cases were more likely to be lower grade (Gleason score $\leq$3+4 = 7; P = 0.01, Fisher's exact test), slightly younger (mean age 60.5 vs. 62.5; $P<0.0001$, Wilcoxon rank sum test) and have lower pre-operative serum PSA levels (7.9 vs. 9.3ng/ml; P = 0.0003, Wilcoxon rank sum test) compared to ERG negative cases. There were no differences in Gleason score distribution, age or pre-operative PSA levels in the SPINK1 positive and negative cases. When cases were grouped for positive staining for either marker vs. no staining for either marker, positive staining results were correlated with lower Gleason score (Gleason score $\leq$3+4 = 7; P = 0.03, Fisher's exact test), age (mean age 60.6 vs. 62.5; P = 0.0001, Wilcoxon rank sum test) and pre-operative serum PSA levels (7.9 vs. 9.4 ng/ml; P = 0.0005, Wilcoxon rank sum test) and this association appeared to be largely driven by ERG positive cases. The presence of extracapsular extension was slightly lower in cases in which either marker was positive (41.4%) compared to cases in which both markers were negative (58.6%) (P = 0.05). However, neither marker alone was associated with extracapsular extension.

ERG/SPINK1 expression and clinical outcomes

In univariate Cox proportional hazards analysis, positive ERG expression was associated with improved RFS (HR = 1.23; P = 0.04), as was strong positive SPINK1 expression

Table 1. Summary of clinical, pathological and staining characteristics.

Variable	Status	Number	Percent
Gleason Score	Missing	10	0.94
	≤6	429	40.21
	3+4	387	36.27
	4+3	133	12.46
	10-Aug	108	10.12
Extracapsular extension	Missing	9	0.84
	Negative	793	74.32
	Positive	265	24.84
Surgical margins	Missing	179	16.78
	Positive	306	28.68
	Negative	582	54.55
Seminal vesicle invasion	Missing	14	1.31
	No	984	92.22
	Yes	69	6.47
ERG staining	Missing	113	10.59
	0	506	47.42
	1	29	2.72
	2	419	39.27
SPINK1 staining	Missing	104	9.75
	0	826	77.41
	1	68	6.37
	2	24	2.25
	3	12	1.12
	4	33	3.09
Recurrence Free Survival	No Event	588	55.11
	Event	479	44.89
Disease Specific Survival	No Event	1013	94.94
	Mets or Ca Death	54	5.06
Overall Survival	Alive	996	93.35
	Dead	71	6.65

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(HR = 3.32; P = 0.004) and positive expression of either marker (HR = 1.33; P = 0.003). However, neither marker, either alone or in combination, was associated with DSS or OS (Table 3). High level expression of ERG (Fig 2A) and SPINK1 (Fig 2B) was associated with improved RFS by Kaplan-Meier analysis, although neither was associated with DSS (Fig 2C and 2D) or OS (not shown).

To evaluate whether either biomarker provided prognostic information independent of clinical variables, we performed multivariate Cox proportional hazards analysis using a backwards elimination procedure to identify the final model for each endpoint (Table 4). For RFS, absent SPINK1 expression was correlated with worse clinical outcome (HR = 2.84; P = 0.02), as were presence of positive surgical margins, seminal vesicle invasion, higher pre-operative PSA and increasing Gleason score. ERG expression was not associated with RFS, DSS or OS. DSS was associated only with Gleason score and pre-operative PSA and OS were associated only with Gleason score and age. The relatively small number of prostate cancer deaths or metastases (54) and deaths from all causes (71) limited our ability to test the association of the biomarkers

Table 2. Summary of ERG, SPINK1, and ERG/SPINK1 by pathological features.

Feature	Status	ERG Neg	ERG Pos	P-value	SPINK1 Neg	SPINK1 Pos	P-value	Both Neg	Either Pos	P-value
Surgical Margin	Positive	168(61.5%)	105(38.5%)	0.08	264(96%)	11(4%)	1.00	158(57.9%)	115(42.1%)	0.13
	Negative	285(55%)	233(45%)		505(96.2%)	20(3.8%)		270(52.1%)	248(47.9%)	
Stage	III/IV	142(61.5%)	89(38.5%)	0.26	227(97.4%)	6(2.6%)	0.31	137(59.3%)	94(40.7%)	0.13
	I/II	302(56.9%)	229(43.1%)		514(95.5%)	24(4.5%)		282(53.1%)	249(46.9%)	
SVinv	Negative	482(54.9%)	396(45.1%)	0.09	856(96.6%)	30(3.4%)	1.00	458(52.2%)	420(47.8%)	0.11
	Yes	41(66.1%)	21(33.9%)		61(96.8%)	2(3.2%)		39(62.9%)	23(37.1%)	
ECE	Negative	385(54.8%)	318(45.2%)	0.18	682(95.9%)	29(4.1%)	0.10	361(51.4%)	342(48.6%)	0.05
	Yes	146(59.8%)	98(40.2%)		241(98.4%)	4(1.6%)		143(58.6%)	101(41.4%)	
Gleason Score	< = 6	190(51.8%)	177(48.2%)	0.01	362(97.1%)	11(2.9%)	0.63	183(49.9%)	184(50.1%)	0.03
	3+4	198(55%)	162(45%)		347(96.1%)	14(3.9%)		186(51.7%)	174(48.3%)	
	4+3	83(66.4%)	42(33.6%)		122(97.6%)	3(2.4%)		80(64%)	45(36%)	
	8–10	61(64.2%)	34(35.8%)		92(94.8%)	5(5.2%)		56(58.9%)	39(41.1%)	

P-values by Fisher's exact test.

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with these endpoints. Conclusions from weighted and unweighted analyses were similar with respect to biomarker effects on survival endpoints.

Discussion

Molecular subtypes of prostate cancer defined by ERG expression do not appear to correlate with clinical outcomes in patients undergoing surgery for localized prostate cancer. On the other hand, we found that high SPINK1 protein expression was associated with lower rates of recurrence after surgery, although SPINK1 overexpression defines only a small subset of prostate cancers (3.4%). ERG and SPINK1 expressing cancers do not appear to be strictly mutually exclusive molecular subtypes, although SPINK1 expression does appear to be uncommon in ERG-expressing cancers. This observation agrees with other studies showing a small subset of tumors expressing high levels of both markers [14, 17].

Studies of the prognostic role of the TMPRSS2:ERG fusion or ERG over-expression have reported associations with worse clinical outcome, improved clinical outcome and a lack of association ([18–26] and summarized in [13] and [27]). In some cases, the discrepant findings can be attributed to small sample sizes or segregation of adverse clinical features in ERG positive tumors or ERG negative tumors by chance. For instance, in our univariate analysis, ERG negative tumors had a slightly worse outcome, but this finding disappeared when we adjusted for age, Gleason score and pre-operative serum PSA levels. While an association between ERG expression and age and serum PSA levels has been observed in previous studies [13, 28] this association is unlikely to reflect prostate cancer biology since the relative frequency of the TMPRSS2:ERG fusions appears to be similar across early stage and metastatic prostate cancer, implying there is no selection of this molecular subtype with progression [29, 30]. It is also possible that the range of associations of the TMPRSS2:ERG fusion or ERG over-expression with prognosis is due to differences in the populations studied or other clinical or pathologic features. For example, ERG fusions and over-expression can vary between different ethnic groups and are less common in transition zone tumors [13, 31, 32]. Prostate cancer outcomes after surgery have been associated with ethnicity and tumor location [33–35]. The size of our cohort and distribution of cases across several institutions, as well as the careful case selection likely minimized these potential biases, and we found no association of ERG expression with clinical outcome. Our data support an emerging consensus that the presence of the TMPRSS2:ERG

Table 3. Univariate Cox proportional hazard models.

Endpoint	Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value	# Event	# Censored	Total # Patients
RFS	ERG	Neg vs. Pos	1.23	1.01	1.49	0.04	435	519	954
	SPINK1	Neg vs. Pos	3.32	1.48	7.42	0.004	438	525	963
	ERG/SPINK1	Neg vs. Pos	1.33	1.1	1.61	0.003	435	519	954
	Margin	Pos vs. Neg	2.03	1.66	2.47	< .0001	395	493	888
	Stage	III/IV vs. I/II	2.4	1.96	2.94	< .0001	385	477	862
	SVinv	No vs. Yes	0.28	0.21	0.38	< .0001	470	583	1053
	ECE	No vs. Yes	0.5	0.41	0.61	< .0001	474	584	1058
	Gleason	3+4 vs. < = 6	1.58	1.27	1.98	0.0001	470	587	1057
		4+3 vs. < = 6	2.7	2.07	3.53	< .0001			
		8–10 vs. < = 6	2.62	1.96	3.52	< .0001			
	Age	1 unit increase	1.01	0.99	1.02	0.43	459	502	961
	Log(pre-op PSA)	1 unit increase	1.96	1.69	2.27	< .0001	431	510	941
DSS	ERG	Neg vs. Pos	1.16	0.65	2.07	0.61	49	899	948
	SPINK1	Neg vs. Pos	NA	NA	NA	0.99	50	907	957
	ERG/SPINK1	Neg vs. Pos	1.32	0.74	2.35	0.35	49	899	948
	Margin	Pos vs. Neg	2.44	1.27	4.69	0.0073	37	847	884
	Stage	III/IV vs. I/II	6.7	3.13	14.33	< .0001	35	821	856
	SVinv	No vs. Yes	0.29	0.15	0.57	0.0004	54	994	1048
	ECE	No vs. Yes	0.39	0.22	0.67	0.0007	52	1000	1052
	Gleason	3+4 vs. < = 6	2.55	1.19	5.47	0.02	53	998	1051
		4+3 vs. < = 6	3.56	1.44	8.82	0.006			
		8–10 vs. < = 6	6.88	3.05	15.56	< .0001			
	Age	1 unit increase	1.02	0.98	1.06	0.3	53	902	955
	Log(pre-op PSA)	1 unit increase	2.12	1.49	3.02	< .0001	47	888	935
OS	ERG	Neg vs. Pos	0.72	0.41	1.26	0.25	49	893	942
	SPINK1	Neg vs. Pos	0.6	0.19	1.93	0.39	49	901	950
	ERG/SPINK1	Neg vs. Pos	0.63	0.36	1.11	0.11	49	893	942
	Margin	Pos vs. Neg	1.67	0.99	2.83	0.06	56	823	879
	Stage	III/IV vs. I/II	2	1.19	3.38	0.01	57	792	849
	SVinv	No vs. Yes	0.4	0.19	0.85	0.02	57	984	1041
	ECinv	No vs. Yes	0.48	0.28	0.81	0.01	56	989	1045
	Gleason	3+4 vs. < = 6	0.93	0.47	1.84	0.83	58	986	1044
		4+3 vs. < = 6	1.27	0.51	3.17	0.61			
		8–10 vs. < = 6	4.14	2.18	7.89	< .0001			
	Age	1 unit increase	1.07	1.03	1.11	0.0011	58	890	948
	Log(pre-op PSA)	1 unit increase	1.65	1.11	2.44	0.01	38	890	928

LCL = Lower Confidence Limit, UCL = Upper Confidence Limit, RFS = Recurrence Free Survival, DSS = Disease Specific Survival, OS = Overall Survival

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fusion or ERG over-expression are not associated with more aggressive prostate cancers [13, 27, 36].

High SPINK1 expression was associated with improved RFS in our cohort. This is in contrast with other reports that report high SPINK1 expression associated with worse RFS or null-association [12, 14, 19, 37–39]. It is unclear why SPINK1 expression shows variable results between studies, although it is likely that the small number of SPINK1 positive cases could lead to imbalances in the distribution of clinical risk factors between studies. Given our finding that

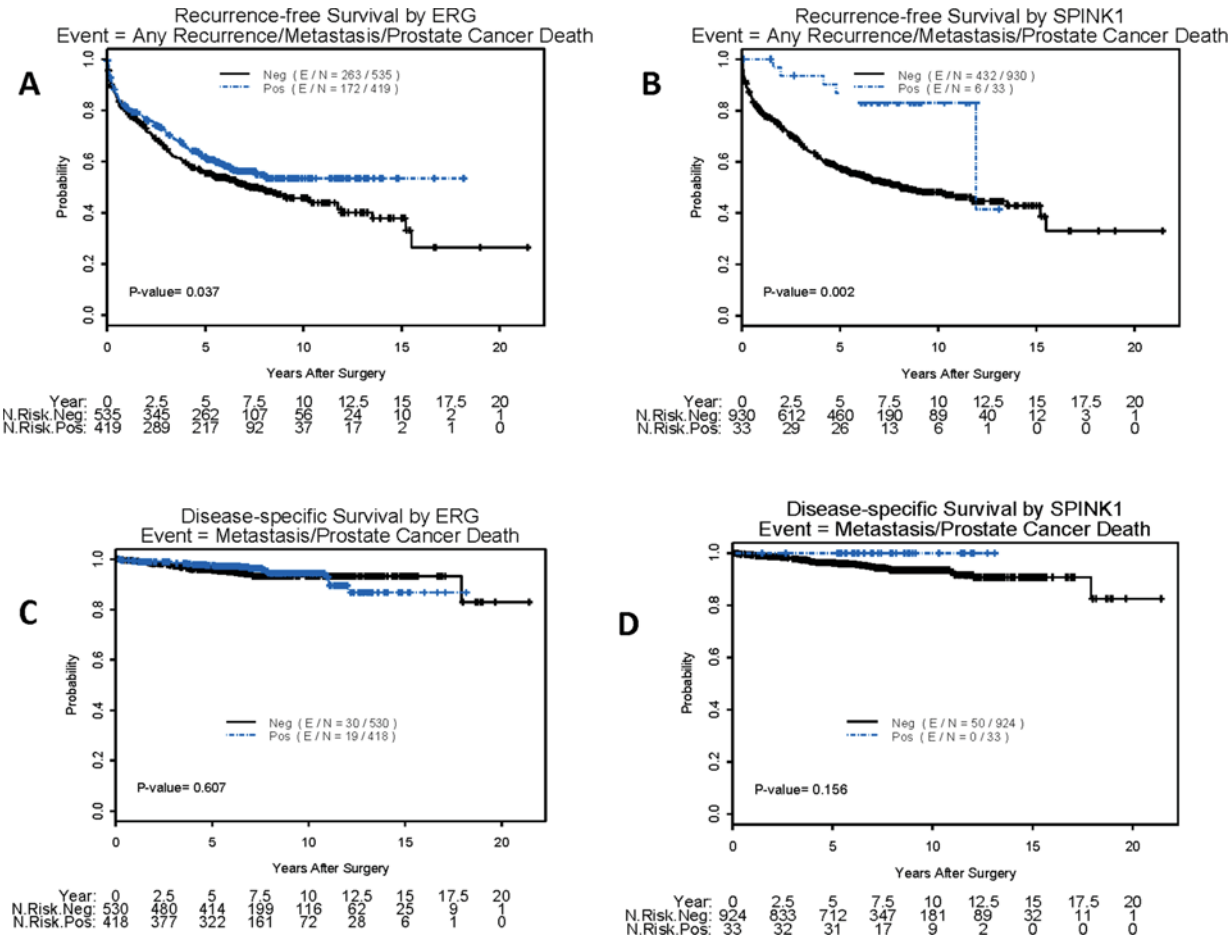

Fig 2. Kaplan-Meier plots of showing the relationship of expression of ERG or SPINK1 and clinical outcome: A) High expression of ERG is associated with improved RFS B) High expression of SPINK1 is associated with improved RFS C) High expression of ERG is not associated with diseases specific survival or development of metastases D) High expression of SPINK1 is not associated with diseases specific survival or development of metastases.

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high expression of SPINK1 is associated with improved outcomes, while others find it associated with worse outcome, our positive association needs to be interpreted with caution.

While ERG status was not prognostic in our cohort, it has been proposed that ERG status might define molecular subtypes that provide context for other biomarkers. For example, PTEN loss has been associated with adverse pathology and worse RFS in ERG overexpressed tumors, but not in ERG negative tumors [17, 23, 40–42]. In addition, increased expression of CRISP3 has been shown to be enriched in high ERG and PTEN expressing tumors and also associated with worse DSS [43]. Low expression of ERG and TERT in urine samples has been associated with improved RFS compared to samples expressing either or both genes [44]. Increased expression of proliferation associated proteins Ki67 and TOP2A has been found to be more highly prognostic in ERG-negative prostate cancers [45]. While loss of expression of p27 has been noted in ERG-negative prostate cancers, p27 loss was not associated with clinical outcomes [46]. Because of the relative infrequency of SPINK1 alterations, it is difficult to assess whether this molecular subclass of tumors can be further subtyped prognostically. ERG and SPINK1 positive tumors have been proposed to describe discrete molecular subtypes of prostate cancer. In our cohort there did not appear to be a significant interaction between these

Table 4. Multivariate Cox proportional hazard models.

Endpoint	Factor	Comparison	Hazard Ratio	95% LCL	95% UCL	P-value
RFS (N = 674,E = 306)	SPINK1	Neg vs. Pos	2.84	1.17	6.90	0.02
	Margin	Pos vs. Neg	1.78	1.41	2.24	<0.0001
	SVinv	Yes vs. No	2.37	1.63	3.43	<0.0001
	Gleason	3+4 vs. < = 6	1.46	1.10	1.95	0.009
		4+3 vs. < = 6	2.09	1.49	2.93	< .0.0001
		8–10 vs. < = 6	1.82	1.26	2.65	0.002
	Log(pre-op PSA)	1 unit increase	1.56	1.31	1.86	< .0.0001
DSS (N = 929,E = 46)	Gleason	3+4 vs. < = 6	2.69	1.11	6.49	0.03
		4+3 vs. < = 6	3.67	1.34	10.07	0.01
		8–10 vs. < = 6	6.27	2.41	16.31	0.0002
	Log(pre-op PSA)	1 unit increase	1.80	1.23	2.64	0.003
OS (N = 940, E = 58)	Gleason	3+4 vs. < = 6	0.88	0.44	1.73	0.71
		4+3 vs. < = 6	1.11	0.44	2.77	0.82
		8–10 vs. < = 6	3.25	1.70	6.24	0.0004
	Age	1 unit increase	1.06	1.02	1.10	0.006

N = total number of patients, E = number of patients with events

LCL = Lower Confidence Limit, UCL = Upper Confidence Limit

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subtype biomarkers. While tumors positive for either of these markers appeared to have improved RFS compared to tumors lacking both, multivariable analysis failed to demonstrate an association between RFS, DSS or OS in marker positive vs. negative cases. Our findings are consistent with a recent publication demonstrating a lack of association with clinical outcome for ERG-positive, ETS-positive, SPINK-positive and marker negative (triple negative) prostate cancers based on gene expression profiling [47]. Much additional work with large clinical datasets, such as ours, will be necessary to test whether molecular subtyping with ERG and SPINK1 will provide clinically or biologically meaningful information in prostate cancer.

While ERG and SPINK1 do not appear to be strong prognosticators, it is possible that they could have other roles as biomarkers, such as in defining molecular subtypes that respond to different therapies (i.e. as predictive biomarkers). For example, in a large cohort (N = 2800) of radical prostatectomy patients, high ERG expression was not correlated with biochemical recurrence, but was correlated with high level expression of the androgen receptor (AR) [36]. This finding suggests that ERG overexpressed tumors might be particularly sensitive to AR inhibition, although this concept has been challenged based on analysis of ERG expression in hormonally treated patients [19]. In addition, TMPRSS2:ERG gene fusions secondary to deletions of chromosome 21q22 and increased copy number of the fusion sequences have been associated with improved progression free survival in patients with castrate resistant prostate cancer treated with abiraterone treatment compared to ERG negative or ERG rearranged tumors [48]. In preclinical studies, SPINK1 expressing tumors have been shown to be susceptible to targeting by anti-SPINK1 antibodies, as well as inhibitions of the EGFR signaling pathway [49]. Therefore, there might be possible roles for assessment of ERG and SPINK1 expression in prostate cancer care in the future.

In summary, high expression of ERG and SPINK1 were associated with improved recurrence free survival in our multi-institutional cohort on univariate analysis. However, only SPINK1 over-expression remained significantly associated with improved RFS in multivariate models that took into account additional clinical and pathological parameters. Furthermore,

neither biomarker was associated with differences in DSS or OS, although the number of events in the cohort was modest. When placed in context of other studies that relate expression of these biomarkers to clinical outcome, it is unlikely that either identifies molecular subtypes that are linked to prognosis. However, it is possible that when combined with other molecular biomarkers, ERG and SPINK1 could be useful in predicting outcome or predicting responses to therapy.

Supporting Information

S1 File. Raw clinical, pathological and staining data from the cohort.
(XLSX)

Author Contributions

Conceived and designed the experiments: JB SH LN LF JS AHC DT PC MG RL DL PN IT LT ZF JM. Performed the experiments: JB SH HB LF JS AHC DT LT JM. Analyzed the data: JB WW HA ZF. Contributed reagents/materials/analysis tools: JB WW SH HA HB LF JS AHC DT LT ZF JM. Wrote the paper: JB WW HA LF PC JM.

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PTEN Loss as Determined by Clinical-grade Immunohistochemistry Assay Is Associated with Worse Recurrence-free Survival in Prostate Cancer

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Abstract

Background—*PTEN* is the most commonly deleted tumor suppressor gene in primary prostate cancer (PCa) and its loss is associated with poor clinical outcomes and *ERG* gene rearrangement.

Objective—We tested whether PTEN loss is associated with shorter recurrence-free survival (RFS) in surgically treated PCa patients with known ERG status.

Design, setting, and participants—A genetically validated, automated PTEN immunohistochemistry (IHC) protocol was used for 1275 primary prostate tumors from the Canary Foundation retrospective PCa tissue microarray cohort to assess homogeneous (in all tumor tissue sampled) or heterogeneous (in a subset of tumor tissue sampled) PTEN loss. ERG status as determined by a genetically validated IHC assay was available for a subset of 938 tumors.

Outcome measurements and statistical analysis—Associations between PTEN and ERG status were assessed using Fisher's exact test. Kaplan-Meier and multivariate weighted Cox proportional models for RFS were constructed.

Results and limitations—When compared to intact PTEN, homogeneous (hazard ratio [HR] 1.66, $p = 0.001$) but not heterogeneous (HR 1.24, $p = 0.14$) PTEN loss was significantly associated with shorter RFS in multivariate models. Among ERG-positive tumors, homogeneous (HR 3.07, $p < 0.0001$) but not heterogeneous (HR 1.46, $p = 0.10$) PTEN loss was significantly associated with shorter RFS. Among ERG-negative tumors, PTEN did not reach significance for inclusion in the final multivariate models. The interaction term for PTEN and ERG status with respect to RFS did not reach statistical significance ($p = 0.11$) for the current sample size.

Conclusions—These data suggest that PTEN is a useful prognostic biomarker and that there is no statistically significant interaction between PTEN and ERG status for RFS.

Patient summary—We found that loss of the PTEN tumor suppressor gene in prostate tumors as assessed by tissue staining is correlated with shorter time to prostate cancer recurrence after radical prostatectomy.

Keywords

Biomarker; *ERG*; Immunohistochemistry; *PTEN*; Prostatic carcinoma; Radical prostatectomy

1. Introduction

PTEN is the most commonly deleted tumor suppressor gene in prostate cancer (PCa) [1–4] and its loss is associated with poor pathologic and clinical outcomes [5–20]. Since the *PTEN* gene is almost always lost by deletion in PCa, fluorescence in situ hybridization (FISH) has

traditionally been used to detect *PTEN* loss and examine its association with outcomes [13,14,16,17,21,22]. However, we and others have demonstrated that *PTEN* loss is commonly subclonal and heterogeneous in primary prostate tumors [23–25], making its detection by FISH or techniques that require nucleic acid extraction technically challenging in some cases. Furthermore, there is emerging evidence that in addition to genetic deletion, PTEN protein levels may be regulated by microRNAs and epigenetic modifications [10,15,18]. To address these issues, we previously optimized and validated an immunohistochemistry (IHC) assay to detect PTEN protein loss [18]. We recently transferred this assay to an automated immunostaining platform that may be run in any Clinical Laboratory Improvement Amendments–certified pathology laboratory.

The *ERG* gene is rearranged in approximately half of all prostate tumors [26,27]. Most studies of surgically treated patients have shown that *ERG* gene rearrangements that lead to increased expression of ERG protein are not associated with poor outcomes on their own [27]. However, the presence or absence of *ERG* rearrangements may modify the association of other risk factors with PCa outcomes [28]. Notably, *PTEN* deletion is more common in *ERG*-rearranged prostate tumors [4,15–17,21,23,25,29–32], and *PTEN* loss almost certainly occurs subsequent to *ERG* rearrangement in most cases [23–25]. This fact led several groups to hypothesize that there may be a synergistic effect of ERG expression and *PTEN* loss on PCa progression [29,30,33]. However, results from human studies have been mixed. While early FISH-based studies suggested that *ERG*-rearranged *PTEN*-deleted tumors may have a higher risk of biochemical recurrence compared to *PTEN*-deleted tumors lacking *ERG* rearrangement [21], the largest FISH-based study did not replicate this finding [17]. In this study we used highly validated, clinical-grade assays to assess the association of PTEN and ERG protein status with recurrence-free survival (RFS) in a large multi-institutional cohort of surgically treated PCa patients. We show that PTEN protein loss is most strongly associated with shorter RFS if the loss is homogeneous in all tumor cores sampled, and that the interaction between PTEN and ERG with respect to RFS did not reach statistical significance.

2. Patients and methods

2.1. Subject selection and tissue microarray (TMA) design

The Canary Foundation retrospective PCa TMA resource has been described in detail elsewhere [34]. In brief, radical prostatectomy (RP) tumor tissue from 1275 patients from six academic centers was selected for the TMA using a quota sampling plan. Recurrent cases of Gleason score 3 + 3 and 3 + 4 and nonrecurrent cases with Gleason score 4 + 4 were oversampled in this cohort. While this strategy diminishes the prognostic significance of Gleason score, it improves power to discover biomarkers that provide prognostic information independent of Gleason score. Each tumor was sampled in triplicate using 1-mm cores. The study was approved by the institutional review board at each participating institution and was covered by a materials transfer agreement between institutions.

The TMA included samples from men with (1) recurrent PCa; (2) nonrecurrent PCa; and (3) unknown outcome because of inadequate follow-up time (ie, censoring). Recurrent PCa was defined as (1) a single serum prostate-specific antigen (PSA) level >0.2 ng/ml more than 8

wk after RP; and/or (2) receipt of salvage or secondary therapy after RP; and/or (3) clinical or radiologic evidence of metastatic disease after RP. Nonrecurrent PCa was defined as disease with none of the indicators of recurrence for at least 5 yr after RP. Patients with no evidence of recurrent PCa but less than 5 yr of follow-up after RP (ie, censored) were also included in the TMA. The median follow-up for patients alive was 7 yr (range 1 d–21 yr).

2.2. IHC assays

PTEN IHC was performed on the Ventana platform (Ventana Discovery Ultra, Ventana Medical Systems, Tucson, AZ, USA) using a rabbit anti-human PTEN antibody (Clone D4.3 XP; Cell Signaling Technologies, Danvers, MA, USA). We previously validated a manual version of this assay using the same primary antibody [18].

PTEN protein status was visually scored by a trained pathologist (T.L.L.) blinded to clinical data. A second reviewer (C.L.M.) independently scored all of the cases for evaluation of interobserver variability in scoring. A tissue core was considered to have PTEN protein loss if the intensity of cytoplasmic and nuclear staining was markedly lower or entirely negative across >10% of tumor cells compared to surrounding benign tissue and/or stroma, which provide internal positive controls [18]. If PTEN was lost in >10% and <100% of the tumor cells sampled in a given core, the core was annotated as showing heterogeneous PTEN loss. Alternatively, if the core showed PTEN loss in 100% of sampled tumor tissue, the core was annotated as showing homogeneous PTEN loss. Cores were scored as having ambiguous PTEN IHC results when the intensity of the tumor cell staining was light or absent in the absence of evaluable internal benign tissue or stromal staining.

For statistical analysis, each tumor was scored for the presence or absence of PTEN loss by summarizing scores for the cores sampled. A tumor was designated as having heterogeneous PTEN loss if at least one tumor core showed heterogeneous PTEN loss (intracore heterogeneity), or alternatively, if at least one core showed heterogeneous or homogeneous PTEN loss and at least one core showed intact PTEN in tumor cells (intercore heterogeneity). A tumor was scored as showing homogeneous PTEN loss if all tumor cores sampled showed homogeneous PTEN loss. Finally, a tumor was scored as having intact PTEN if all sample tumor cores showed intact PTEN.

ERG IHC was performed using a commercial rabbit monoclonal antibody to ERG (clone EPR3864; 1:100; Epitomics, Burlingame, CA, USA) as previously described [35]. One set of TMAs from a single institution (Eastern Virginia Medical School) was excluded because of technically insufficient staining. ERG staining was manually scored for each individual core as follows: 0 = no staining; 1 = faint nuclear staining visualized at high-power magnification; and 2 = strong nuclear reactivity easily seen at low-power magnification. In the current study, a tumor was considered ERG-positive in any tissue core showing strong nuclear reactivity for ERG. It has been shown that these dichotomous ERG scoring criteria correlate to fusion status [35,36].

2.3. Statistical analysis

Fisher's exact test was used to assess association between PTEN IHC and ERG status. Kaplan-Meier estimates of RFS were plotted by biomarker. An RFS event is defined as any

recurrence (clinical, biochemical, or salvage therapy), metastasis, or PCa death after surgery. A Cox proportional hazards model was used to correlate multiple factors and biomarkers with RFS. All tests were two-sided and $p < 0.05$ or less was considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC, USA). Graphs were generated using Spotfire S+ version 8 (TIBCO, Palo Alto, CA, USA).

3. Results

Of the 1275 patients with tissue sampled for the TMAs, 1095 (86%) had evaluable PTEN status by IHC and 180 (14%) had missing data. Among the latter, 30/180 (17%) had ambiguous immunostaining results and 150/180 (83%) lacked tumor tissue in the TMA cores sampled. Of the tumors with evaluable staining, 258/1095 (24%) showed any PTEN protein loss, comprising 150 (14%) with heterogeneous PTEN loss (in some but not all tumor tissue sampled) and 108 (10%) with homogeneous PTEN loss (in all tumor tissue sampled; Fig. 1). The remaining 837/1095 (76%) cases had intact PTEN protein according to IHC for all tumor tissue sampled. Of the 150 cases with heterogeneous PTEN loss, 46 (31%) had only intercore heterogeneity (some cores with total loss and some with intact PTEN), nine (6%) had only intracore heterogeneity, and 95 (63%) had both intracore and intercore heterogeneity. A second reviewer scored all TMAs for evaluation of interobserver variability in PTEN IHC scoring. There was very low interobserver variability between the two independent reviewers, with 96.4% agreement over 2783 cores ($\kappa = 0.905$; 95% CI=0.887–0.923). Data for ERG in the cohort overall are reported elsewhere (Brooks JD et al, PLOS ONE, in press). ERG immunostaining results were available for 938 of the 1095 cases with interpretable PTEN IHC results (86%). Of these 938 cases, 401 (43%) were ERG-positive and the remainder were ERG-negative. PTEN loss (homogeneous or heterogeneous) was relatively enriched among the ERG-positive tumors, with 132/401 (33%) of ERG-positive tumors showing any PTEN loss compared to 99/537 (18%) of ERG-negative tumors ($p < 0.0001$; Table 1). ERG-negative tumors with any PTEN loss were slightly more likely to have homogeneous PTEN loss (48/99, 48%) than ERG-positive tumors with any PTEN loss (49/132, 37%), although this did not reach statistical significance ($p = 0.11$, Fisher's exact test).

PTEN IHC status was associated with a number of clinicopathologic factors, including Gleason score and pathologic stage. Because information on pelvic lymph node status was missing for nearly 40% of the cohort, we were not able to assess correlation of PTEN loss with this parameter. Homogeneous PTEN loss was seen in only 4% of tumors with Gleason score ≤6, compared to 18% of tumors with Gleason score 8–10 ($p < 0.0001$; Table 2). PTEN loss was also associated with extraprostatic extension ($p < 0.0001$) and seminal vesicle invasion ($p = 0.0009$), and was thus associated with overall pathologic stage ($p < 0.0001$). However, PTEN loss was not associated with preoperative PSA, patient age, or surgical margin status (Tables 2 and 3). In univariate models, homogeneous PTEN loss was significantly associated with shorter RFS compared to intact PTEN (hazard ratio [HR] 2.04; $p < 0.0001$) and heterogeneous PTEN loss (HR 1.43; $p = 0.03$; Table 4, Fig. 2A). When grouped together, any PTEN loss (heterogeneous or homogeneous) was significantly associated with shorter RFS compared to intact PTEN (HR 1.66, $p < 0.0001$; Table 4, Fig.

2B). When stratified by ERG status, any PTEN loss (heterogeneous or homogeneous) was significantly associated with shorter RFS for both ERG-positive (HR 2.06, $p < 0.0001$) and ERG-negative tumors (HR 1.62, $p = 0.001$; Table 4, Fig. 3).

Multivariate models were constructed for a subset of 808 patients with complete clinicopathologic information available. There was no difference between patients included in these models and those excluded because of incomplete information for any clinicopathologic variable measured (Supplementary Tables 1 and 2). In multivariate models, homogeneous PTEN loss was associated with shorter RFS compared to intact PTEN (HR 1.66, $p = 0.001$; Table 5). Heterogeneous PTEN loss showed a nonsignificant trend towards shorter RFS compared to intact PTEN (HR 1.24, $p = 0.14$; Table 5). To assess the additive value of PTEN for RFS prediction when combined with clinicopathologic factors, area under the receiver operating characteristic curve (AUC) plots were constructed (Supplementary Fig. 1). Inclusion of clinicopathologic factors yielded AUC of 0.72, while addition of two and three PTEN IHC status categories increased AUC to 0.73 and 0.74, respectively.

Multivariate models were also constructed for ERG-positive and ERG-negative tumors separately. Among ERG-positive tumors, homogeneous (HR 3.07, $p < 0.0001$) but not heterogeneous PTEN loss (HR 1.46, $p = 0.10$) was significantly associated with shorter RFS compared with intact PTEN. Among ERG-negative tumors, PTEN loss did not reach significance for inclusion in the final model ($p = 0.08$), although the effect of PTEN loss was in the same direction as seen for the ERG-positive group. The interaction term between PTEN and ERG status did not reach statistical significance in a multivariate Cox model for RFS ($p = 0.11$), although post hoc bootstrapping simulations indicated that at least 1000 patients are required to detect an interaction with 80% power. In multivariate models in which homogeneous and heterogeneous PTEN loss were grouped together, any PTEN loss was associated with shorter RFS (HR 1.40, $p = 0.004$; Table 6). When ERG-positive and ERG-negative tumors were considered separately in multivariate models, the association between any PTEN loss and shorter RFS was significant for ERG-positive tumors (HR 1.98, $p = 0.0003$) and nonsignificant for ERG-negative tumors, so was not included in the final model. When PTEN status was modeled as intact or loss, the interaction term between PTEN and ERG status was not statistically significant for RFS ($p = 0.25$).

4. Discussion

There is a growing need for biomarkers that help to distinguish indolent from aggressive prostate tumors and add to current clinicopathologic risk stratification measures. We recently developed and validated an IHC assay to assess PTEN protein loss in PCa [18]. The original assay involved manual staining of slides, but we have now adapted this assay for automated performance on a Ventana autostainer system and demonstrated equivalence to the manual assay. In a subset of 551 tumors for which IHC (by the automated assay) and FISH data were available [22], we found that intact PTEN immunostaining was 91% specific for the absence of *PTEN* gene deletion by FISH, and 98% and 62% sensitive for detection of homozygous and hemizygous gene deletion, respectively, by FISH [37].

Using manual IHC, our group previously demonstrated that PTEN protein loss is associated with higher risk of biochemical recurrence in a nested case-control cohort of surgically treated patients [20]. Similar to the current findings with the automated protocol, PTEN loss correlated with higher Gleason grade and stage, and homogeneous PTEN loss was independently associated with biochemical recurrence in multivariate models with HR of approximately 2. Of note, heterogeneous PTEN loss (in some but not all tumor tissue sampled) was a weaker prognostic indicator compared to homogeneous loss, as seen in the current study. Some cases of heterogeneous loss will likely be missed when the IHC assay is applied to prostate biopsies because of sampling error (and may have been similarly missed in the current TMA sampling). However, since heterogeneous loss is more weakly associated with poor outcomes, these false negatives may be less clinically significant. It remains unclear why homogeneous PTEN loss is more tightly associated with shorter RFS. Homogeneous loss of PTEN protein may signify increased selection for (and expansion of) a single PTEN-null clone, a finding that has been associated with PCa progression in a recent single-cell analysis [38]. Equally plausible is the possibility that tumors with a higher mass of PTEN-null cells have a higher risk of local or disseminated spread for stochastic reasons. The current study adds insights to work identifying a putative interaction between PTEN loss and *ERG* rearrangements. Mouse models have suggested that PTEN loss and *TMPRSS2:ERG* gene rearrangement synergize to drive cell migration and invasion, perhaps explaining the tendency towards co-occurrence in human PCa [29,30]. Furthermore, in the mouse prostate, ERG expression may restore decreased androgen signaling due to reciprocal feedback between PI3K and androgen receptor in the context of PTEN loss [33]. At least four studies have examined the interaction of PTEN and ERG in association with PCa progression in clinical series. The first study to explore the interaction between ERG and PTEN used FISH to assess *PTEN* gene status in 125 patients [21] and found that *PTEN* loss was more strongly associated with biochemical recurrence after RP among ERG-positive compared to ERG-negative tumors. However, a larger study of 1895 patients [17] found no influence of ERG status on the association of *PTEN* deletion assessed by FISH with postoperative biochemical recurrence, and this result was replicated in an expanded cohort including more than 5000 patients [39]. In a study of 262 patients [40], loss of PTEN protein expression by IHC was more strongly associated with biochemical recurrence among ERG-positive compared to ERG-negative tumors. Similar findings have been reported for a cohort of patients treated with brachytherapy [41]. Only one study has examined the interaction of PTEN and ERG and their association with PCa-specific mortality in a cohort of 308 patients managed conservatively [16]. Interestingly, *PTEN* deletion detected by FISH was associated with higher risk of PCa mortality among ERG-negative but not ERG-positive tumors. However, in a subsequent study of 652 patients (including the original 308 patients), the authors failed to validate this interaction between *PTEN* deletion and ERG status with respect to PCa death [42].

Taken together, our study and previous work suggest that PTEN loss is associated with biochemical recurrence in both ERG-positive and ERG-negative tumors. In our study, the interaction term for PTEN and ERG status with respect to RFS did not reach statistical significance in the Cox models. While post hoc power calculations indicate that we would have needed at least 1000 samples to achieve 80% power to detect a significant interaction,

such analyses must be interpreted with caution. While some prior studies have found that PTEN loss is associated with poor prognosis only for ERG-positive tumors, this finding could be because of the relative enrichment of PTEN loss among ERG-positive tumors. Indeed, this may be why PTEN loss was not significant in the final multivariate models for ERG-negative tumors, but was significant for ERG-positive tumors. In previous studies, only between five and 19 ERG-negative tumors with PTEN loss were available for follow-up [21,40,41], so the studies may also have been underpowered for observation of an association with outcome in this subgroup. By contrast, the largest FISH-based study that found no effect of ERG status on the association of PTEN loss with progression examined 97 and 356 ERG-negative PTEN-loss tumors in the original and expanded series [17,39].

There are a number of important limitations to the current study. Because of the multi-institutional design, some data for the cohort are incomplete, including the lymph node status of patients (missing for >40% of cases) and racial and family history information. In addition, the outcome measured in the current study is RFS rather than PCa-specific mortality. Of patients experiencing biochemical recurrence (as was seen in the majority of the recurrent cases in the current study), only a minority will die from PCa, so this remains a surrogate outcome measure with well-described limitations. Finally, the degree to which PTEN adds to established clinicopathologic factors for prediction of prognosis in the RP setting remains unclear. Receiver operating characteristic analysis demonstrated that PTEN shifted the area under the curve (AUC) from 0.72 (with clinicopathologic factors alone) to 0.74 (combined three-category PTEN score and clinicopathologic factors). This effect size is similar to that observed for newly available genomic classifiers such as Decipher in the RP setting [43]. However, as seen in studies of genomic classifiers, even marginal shifts in AUC can have a significant impact on decision curve analysis. Perhaps more importantly, complete grading and pathologic staging information is not available in the setting of needle biopsies, and thus biomarkers such as PTEN are likely have more added value for prediction of prognosis.

5. Conclusions

Using a highly validated and automated IHC assay for a diverse and multi-institutional set of PCa tumors, we found that homogeneous rather than heterogeneous PTEN protein loss is most strongly associated with a higher risk of recurrence after RP, even after adjusting for other clinicopathologic parameters. In univariate analyses, PTEN loss was associated with poor outcomes among both ERG-positive and ERG-negative tumors and we did not find evidence for a statistically significant interaction between PTEN and ERG status in predicting RFS in multivariate models. If reproduced in additional cohorts, these data suggest that PTEN IHC may be a simple and relatively inexpensive test to aid in stratification of PCa risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Take Home Message

We used highly validated, clinical-grade assays to assess the association of PTEN and ERG protein status with recurrence-free survival (RFS) in a large multi-institutional cohort of surgically treated prostate cancer patients. We show that PTEN protein loss is most strongly associated with shorter RFS if the loss is homogeneous in all tumor tissue sampled. In addition, we demonstrate that there is not a statistically significant interaction between PTEN and ERG with respect to RFS.

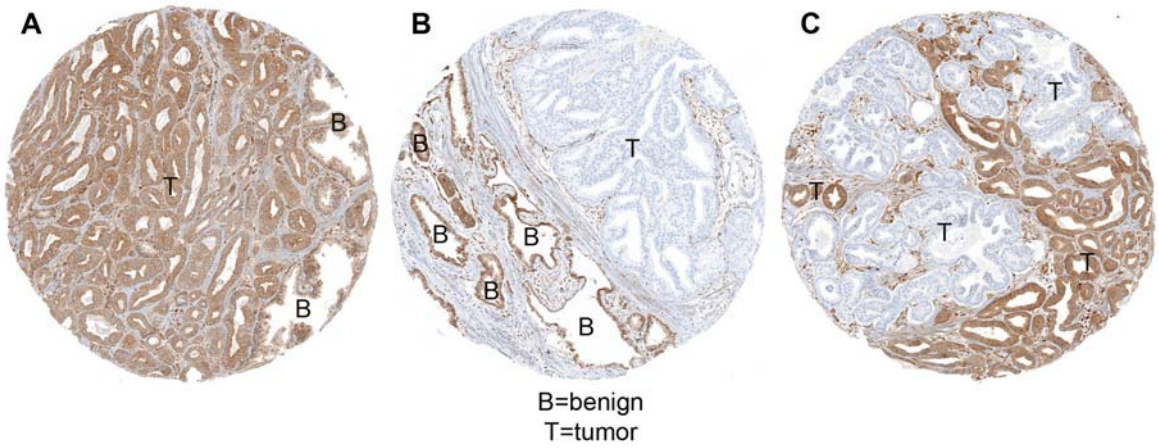

Fig. 1.

PTEN immunostaining examples from Canary the cohort (magnification 200×). (A) Intact PTEN. Tumor tissue has similar immunostaining intensity to surrounding benign tissue. (B) Homogeneous PTEN protein loss. All tumor tissue shows PTEN protein loss, with intact PTEN staining in surrounding benign tissue. (C) Heterogeneous PTEN protein loss. A subset of tumor tissue shows PTEN protein loss, while other intermingled tumor tissue shows intact immunostaining. T = tumor; B = benign.

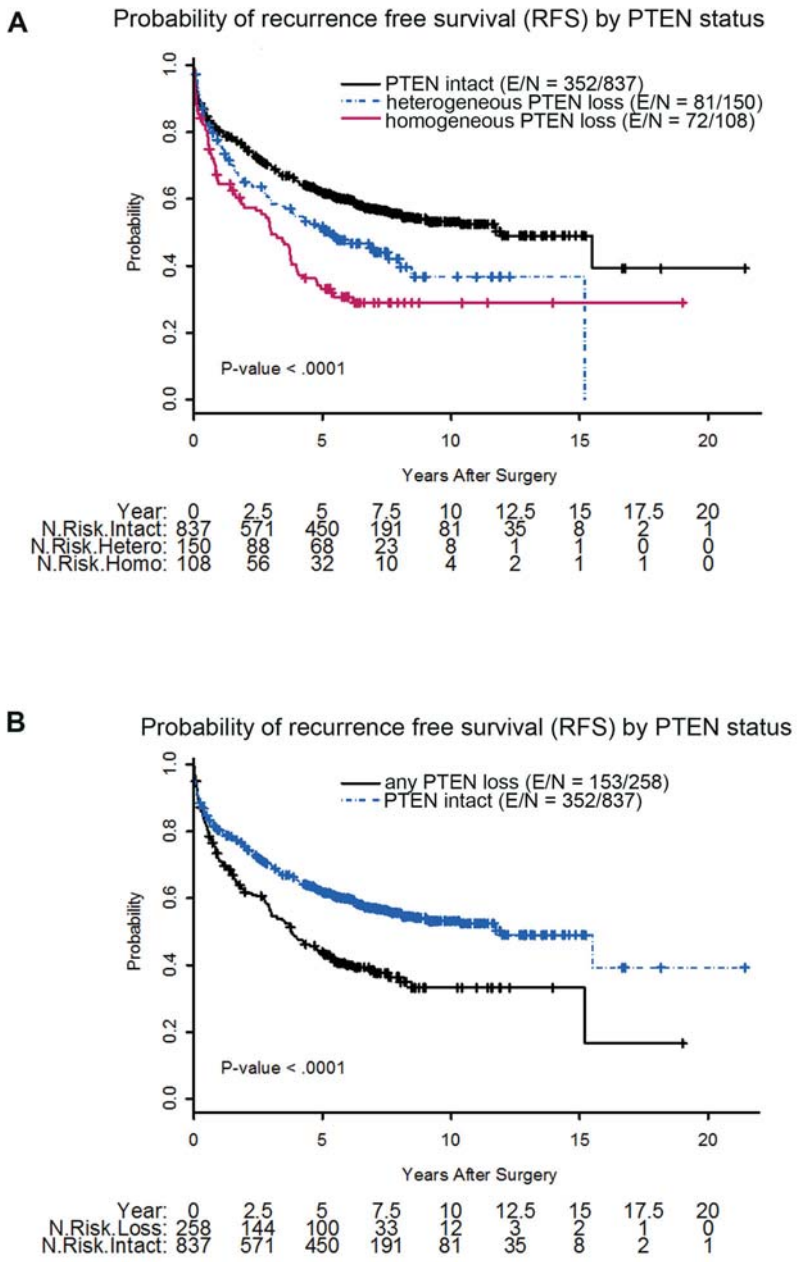

Fig. 2.

Kaplan-Meier probability of recurrence-free survival stratified by (A) homogeneous PTEN loss, heterogeneous PTEN loss, and intact PTEN; and (B) any PTEN loss (homogeneous or heterogeneous) and intact PTEN. N = total number of patients; E = events; hetero = heterogeneous; homo = homogeneous.

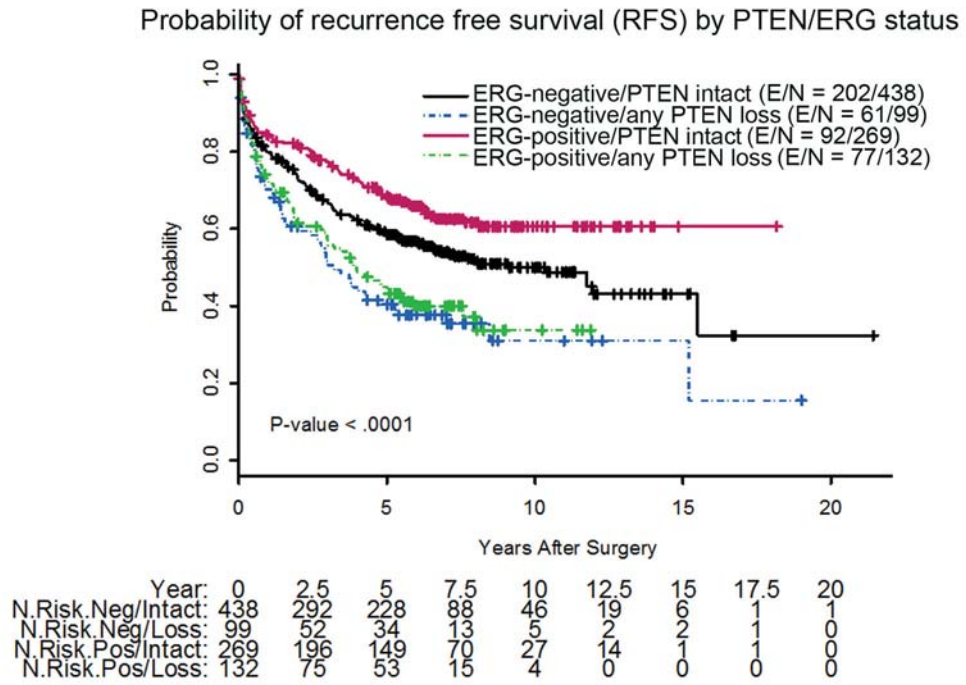

Fig. 3.

Kaplan-Meier probability of recurrence-free survival stratified by PTEN and ERG status. N = total number of patients; E = events; ERG^{-} = ERG-negative; ERG^{+} = ERG-positive.

Table 1

Summary of PTEN immunohistochemistry results stratified by ERG status

	ERG status, *n* (%)	
	Negative	**Positive**
Intact PTEN	438 (81.6)	269 (67.1)
Heterogeneous PTEN loss	51 (9.5)	83 (20.7)
Homogeneous PTEN loss	48 (8.9)	49 (12.2)

Table 2

Association of PTEN immunohistochemistry status with clinicopathologic factors

	PTEN status, *n* (%)				*p* value[a]
	Missing	**Intact**	**Heterogeneous loss**	**Homogeneous loss**	
Margins					
Missing	23 (12.9)	125 (69.8)	20 (11.2)	11 (6.2)	
Positive	53 (13.8)	253 (65.7)	44 (11.4)	35 (9.1)	0.9
Negative	104 (14.6)	459 (64.6)	86 (12.1)	62 (8.7)	
Pathologic stage					
Missing	22 (10.6)	142 (68.6)	25 (12.1)	18 (8.7)	
III/IV	48 (13.8)	206 (59.2)	43 (12.4)	51 (14.7)	<0.0001
I/II	110 (15.3)	489 (67.9)	82 (11.4)	39 (5.4)	
Seminal vesicle invasion					
Missing	0 (0.0)	15 (88.2)	0 (0.0)	2 (11.8)	
No	169 (14.4)	780 (66.3)	137 (11.6)	91 (7.7)	0.0009
Yes	11 (13.6)	42 (51.9)	13 (16.1)	15 (18.5)	
Extraprostatic extension					
Missing	3 (17.7)	12 (70.6)	0 (0.0)	2 (11.8)	
No	124 (14.1)	601 (68.5)	101 (11.5)	51 (5.8)	<0.0001
Yes	53 (13.9)	224 (58.8)	49 (12.9)	55 (14.4)	
Gleason score					
Missing	3 (30.0)	5 (50.0)	2 (20.0)	0 (0.0)	
≤6	98 (17.9)	392 (71.4)	35 (6.4)	24 (4.4)	<0.0001
3 + 4	51 (11.1)	285 (62.2)	79 (17.3)	43 (9.4)	
4 + 3	13 (9.1)	96 (67.1)	14 (9.8)	20 (14.0)	
8–10	15 (13.0)	59 (51.3)	20 (17.4)	21 (18.3)	

[a] χ^2 test.

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Table 3

Association of PTEN immunohistochemistry status with age and preoperative PSA

	n	Mean	SD	Minimum	Median	Maximum	*p* value[a]
Age							
Missing PTEN	174	62.2	7.1	42	63	80	
Intact PTEN	761	61.1	7.2	35	62	77	0.34
Heterogeneous PTEN loss	137	62.3	6.7	44	63	76	
Homogeneous PTEN loss	97	61.3	6.9	45	61	75	
Preoperative PSA							
Missing PTEN	158	8.7	7.1	0.98	6.62	52.6	
Intact PTEN	752	8.5	8.1	0.02	6.32	124	0.6
Heterogeneous PTEN loss	139	8.5	7.9	0.71	6.4	64.85	
Homogeneous PTEN loss	98	9.9	11.2	1	6.85	78.3	

PSA = prostate-specific antigen; SD = standard deviation.

[a]Kruskal-Wallis test.

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Table 4

Summary of univariate Cox models correlating PTEN immunohistochemistry status with recurrence-free survival (RFS)

Comparison	HR (95% CI)	*p* value	Events	Censored	Total
PTEN status					
Homogeneous loss vs intact	2.04 (1.59–2.63)	<0.0001	500	590	1095
Homogeneous vs heterogeneous loss	1.43 (1.04–1.96)	0.03			
Any loss vs intact	1.66 (1.37–2.01)	<0.0001	505	590	1095
ERG-positive PTEN status					
Any loss vs intact	2.06 (1.52–2.90)	<0.0001	169	232	401
ERG-negative PTEN status					
Any loss vs intact	1.62 (1.22–2.16)	0.001	263	274	537

HR = hazard ratio; CI = confidence interval.

Table 5

Multivariate Cox proportional hazards models for recurrence-free survival in association with homogeneous/heterogeneous PTEN loss by ERG status

Model and factor	Comparison	HR (95% CI)	*p* value
All patients			
(*n* = 808, 371 events)			
log(PSA)	1-unit increase	1.44 (1.22–1.70)	<0.0001
PTEN status	Homogeneous loss vs intact	1.66 (1.22–2.24)	0.001
	Heterogeneous loss vs intact	1.24 (0.93–1.65)	0.14
Margins	Positive vs negative	1.73 (1.39–2.16)	<0.0001
Seminal vesicle invasion	Yes vs no	1.93 (1.38–2.71)	0.0001
Extraprostatic extension	Yes vs no	1.30 (1.03–1.63)	0.03
Gleason score	3 + 4 vs 6	1.15 (0.89–1.48)	0.29
	4 + 3 vs 6	1.87 (1.38–2.54)	<0.0001
	8–10 vs 6	1.50 (1.06–2.11)	0.02
ERG-positive			
(*n* = 284, 120 events)			
log(PSA)	1-unit increase	1.56 (1.17–2.07)	0.002
PTEN status	Homogeneous loss vs intact	3.07 (1.94–4.84)	<0.0001
	Heterogeneous loss vs intact	1.46 (0.93–2.30)	0.10
Margins	Positive vs negative	1.88 (1.30–2.72)	0.0008
Seminal vesicle invasion	Yes vs no	3.55 (1.85–6.79)	0.0001
ERG-negative			
(*n* = 454, 220 events)			
log(PSA)	1-unit increase	1.51 (1.22–1.86)	0.0001
Gleason score	3 + 4 vs 6	1.24 (0.89–1.73)	0.20
	4 + 3 vs 6	2.01 (1.37–2.96)	0.0004
	8–10 vs 6	1.78 (1.16–2.72)	0.008
Margins	Positive vs negative	1.82 (1.38–2.41)	<0.0001
Extraprostatic extension	Positive vs negative	1.45 (1.09–1.92)	0.01

HR = hazard ratio; CI = confidence interval; PSA = prostate-specific antigen.

Table 6

Multivariate Cox proportional models for recurrence-free survival in association with any PTEN loss by ERG status

Model and factor	Comparison	HR (95% CI)	*p* value
All patients			
(*n* = 808, 371 events)			
log(PSA)	1-unit increase	1.44 (1.22–1.70)	<0.0001
PTEN status	Any loss vs. intact	1.40 (1.12–1.77)	0.004
Margins	Positive vs negative	1.73 (1.39–2.16)	<0.0001
Extraprostatic extension	Yes vs no	1.32 (1.06–1.66)	0.02
Seminal vesical invasion	Yes vs no	1.95 (1.39–2.74)	0.0001
Gleason score	3 + 4 vs ≤6	1.14 (0.89–1.47)	0.31
	4 + 3 vs ≤6	1.88 (1.38–2.55)	<.0001
	8–10 vs ≤6	1.47 (1.04–2.07)	0.03
ERG-positive			
(*n* = 284, 120 events)			
log(PSA)	1-unit increase	1.60 (1.19–2.14)	0.002
PTEN status	Any loss vs intact	1.98 (1.37–2.87)	0.0003
Margins	Positive vs negative	1.89 (1.30–2.73)	0.0008
Seminal vesical invasion	Yes vs no	2.92 (1.54–5.54)	0.001
ERG-negative			
(*n* = 454, 220 events)			
log(PSA)	1-unit increase	1.51 (1.22–1.86)	0.0001
Margins	Positive vs negative	1.82 (1.38–2.41)	<0.0001
Extraprostatic extension	Yes vs no	1.45 (1.09–1.92)	0.01
Gleason score	3 + 4 vs ≤6	1.24 (0.89–1.73)	0.20
	4 + 3 vs ≤6	2.01 (1.37–2.86)	0.0004
	8–10 vs ≤6	1.78 (1.16–2.72)	0.008

HR = hazard ratio; CI = confidence interval; PSA = prostate-specific antigen.

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ORIGINAL ARTICLE

Prognostic value of Ki67 in localized prostate carcinoma: a multi-institutional study of >1000 prostatectomies

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BACKGROUND: Expanding interest in and use of active surveillance for early state prostate cancer (PC) has increased need for prognostic biomarkers. Using a multi-institutional tissue microarray resource including over 1000 radical prostatectomy samples, we sought to correlate Ki67 expression captured by an automated image analysis system with clinicopathological features and validate its utility as a clinical grade test in predicting cancer-specific outcomes.

METHODS: After immunostaining, the Ki67 proliferation index (PI) of tumor areas of each core (three cancer cores/case) was analyzed using a nuclear quantification algorithm (Aperio). We assessed whether Ki67 PI was associated with clinicopathological factors and recurrence-free survival (RFS) including biochemical recurrence, metastasis or PC death (7-year median follow-up).

RESULTS: In 1004 PCs (~4000 tissue cores) Ki67 PI showed significantly higher inter-tumor (0.68) than intra-tumor variation (0.39). Ki67 PI was associated with stage ($P < 0.0001$), seminal vesicle invasion (SVI, $P = 0.02$), extracapsular extension (ECE, $P < 0.0001$) and Gleason score (GS, $P < 0.0001$). Ki67 PI as a continuous variable significantly correlated with recurrence-free, overall and disease-specific survival by multivariable Cox proportional hazard model (hazards ratio (HR) = 1.04-1.1, $P = 0.02$–0.0008). High Ki67 score (defined as $\geqslant 5\%$) was significantly associated with worse RFS (HR = 1.47, $P = 0.0007$) and worse overall survival (HR = 2.03, $P = 0.03$).

CONCLUSIONS: In localized PC treated by radical prostatectomy, higher Ki67 PI assessed using a clinical grade automated algorithm is strongly associated with a higher GS, stage, SVI and ECE and greater probability of recurrence.

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INTRODUCTION

Differentiating indolent from aggressive prostate cancer (PC) is a major priority given the high prevalence of PC in the aging population and the current magnitude of its overtreatment.[1–3] Although clinical and pathological assessments of tumor characteristics provide prognostic information, there is a broad spectrum of outcomes in individual patients. Substantial investments have been made in identifying biomarkers related to PC behavior, although to date no tissue-based markers have been incorporated in routine clinical practice due to conflicting data, lack of independence from other well established clinicopathological characteristics and a paucity of appropriately designed and powered validation and standardization studies.

The Canary Tissue Microarray of Prostate cancer outcomes (CTMAP) was designed with the primary objective of validating promising candidate biomarkers reported to predict PC recurrence at the time of radical prostatectomy (RP).[4] The CTMAP model of a case–cohort study was designed to allow validation of a biomarker with approximately twofold sensitivity greater than Gleason score (GS) in predicting PC recurrence. Such a marker would likely benefit clinical practice. Oversampling of recurrent low-grade tumors and non-recurrent high-grade tumors in CTMAP decreases the influence of GS, allowing unbiased validation of independent prognostic biomarkers. This large and well-annotated tissue resource of more than 1300 randomly selected RP specimens with prolonged follow-up from six academic institutions also reflects the heterogeneity of PC and spectrum of patient management in North America.

Comprehensive literature reviews and a recent meta-analysis of prognostic PC biomarkers indicates that immunohistochemical measurement of Ki67 (*MKI67/MIB-1*) expression is the tissue biomarker with the most consistent association with the clinical outcomes of PC.[5–7] Ki67 has provided independent prognostic value in prostate needle biopsies, transurethral prostate resections and prostatectomy specimens[1,8,9] including independent associations with biochemical and clinical recurrence regardless of treatment.[9] In addition, Ki67 is an attractive biomarker from a technical perspective due to ease of interpretation with moderate to high intra- and inter-observer reproducibility, relatively high tolerance to typical preanalytical variability, universal use and availability across diagnostic laboratories, and the frequent presence of internal positive controls within sampled tissues.[3,7,10,11] As a marker of tumor proliferation, Ki67 has been successfully used in routine pathology practice for differential diagnoses, grading, prognostication and assessment of treatment responses for multiple neoplasms including endocrine and neuroendocrine neoplasms,[12,13] breast cancer,[10,14] trophoblastic tumors, lymphomas, soft tissue and brain tumors.[15–17]

In view of the need for biomarkers that aid in the clinical management of men with PC, we sought to determine if automated detection of Ki67 immunohistochemical staining,

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reported as a percentage of cells expressing Ki67 as a measure of cell proliferation, associated with a specific adverse PC outcome: recurrence after primary therapy. We also sought to determine if Ki67 provided information independent of other risk features and if the magnitude of this additional information was sufficient to impact clinical practice.

MATERIALS AND METHODS

The Canary tissue microarray prostate cancer resource

This study utilized tissue microarrays (TMA) comprised of tissue samples from over 1300 randomly selected participants treated for PC with RP at six institutions between 1995 and 2004.[4] The cohort included approximately equal numbers of samples from men with biochemically recurrent and non-recurrent PC after 5 or more years of follow-up. Recurrent PC was defined by[1] a single serum PSA level >0.2 ng/ml more than 8 week after RP and/or[2] receipt of salvage or secondary therapy after RP and/or[3] clinical or radiologic evidence of metastatic disease after RP. Median follow-up was 7 years (range: 1 day - 21.4 years).

The TMA was constructed to assess biomarkers that provide prognostic information independent of clinical and pathological information. As GS is a powerful predictor of outcome, we oversampled recurrent cases of GS 3+3 and 3+4 as well as non-recurrent cases with GS 4+4. This strategy diminishes the prognostic significance of GS and allows for the validation of biomarkers that correlate with PC clinical outcomes, independent of GS. All six participating sites contributed approximately 200 formalin-fixed and paraffin embedded RP specimens each, which were distributed on 33 TMA blocks. Each TMA block with 11×16 layouts was fabricated from 42 RP specimens and eight normal control tissues including tonsil, prostate, kidney, colon and liver. Each PC, sampled with a 1 mm puncher, was represented by three cores obtained from the highest-grade cancer in the dominant tumor nodule. In addition, one core was obtained from histologically benign prostatic tissue of every patient.

This study was conducted with a multi-institutional agreement and approvals from the institutional review boards at University of Washington, Stanford University, University of British Columbia, University of California San Francisco, University of Texas Health Sciences Center San Antonio, Veterans Affairs Puget Sound Health Care System and Fred Hutchinson Cancer Research Center (FHCRC; Coordinating Center). De-identified demographic, clinical and pathological data are maintained in a central data repository at FHCRC managed by the Coordination Center.

Ki67 immunostaining

Unstained 4 μm TMA sections were deparaffinized on an automated immunostainer (Bond III, Leica Biosystems, Wetzlar, Germany) using a proprietary Bond Dewax solution. Three applications of the Dewax solution were followed by three applications of 100% ethanol and then three applications of Bond Wash solution. The immunostaining was performed in a CAP certified diagnostic immunohistochemistry laboratory according to a standardized protocol. In brief, antigen retrieval was performed on Bond III using ER2 buffer (pH 9.0) for 30 min. After rinsing and endogenous peroxidase blocking, a post primary IgG linker was applied followed by several rinses with the Bond wash solution and a de-ionized water rinse. The slides were incubated for 15 min with a mouse monoclonal IgG1 antibody against Ki67 (clone MIB-1, dilution 1:200, Cat# M7240, DAKO, Carpinteria, CA, USA). Slides were then rinsed multiple times with Bond Wash solution, a polymer anti-mouse Poly-HRP-IgG was applied, and slides were incubated for 8 min with polymer detection reagent (Bond Polymer Refine Detection kit, Leica). This was followed by multiple rinses, reacted with 3,3"-diaminobenzidine tetrahydrochloride chromogen for 10 min, and counterstained with hematoxylin for 5 min. Negative controls for the immunostaining were prepared by omitting the primary antibody step and substituting it with non-immune mouse serum. Normal tonsil tissue cores present on each TMA section served as internal positive controls for Ki67 staining.

Automated Image analysis

The TMA slides were scanned on Aperio ScanScope AT (Aperio Technologies, Vista, CA, USA) at $\times200$ magnification. High-resolution digital image files were created and saved in the web-based Spectrum Plus (Aperio Technologies) digital slide manager and segmented using TMALab to further automate the analysis of the individual gridded tissue cores. The tumor areas were manually annotated by an experienced genitourinary pathologist (MT) to maximally exclude non-tumor stroma, benign glands and inflammatory cells (Figure 1). The Ki67 staining of the cells comprising the tumor areas of each core was determined using a nuclear quantification algorithm, which was tuned by an experienced user (MT) to allow reliable identification of all Ki67-positive nuclei and hematoxylin-counterstained negative nuclei.[18] The threshold for size and shape of tumor cells was manually calibrated to maximally exclude stromal cells and lymphocytes as previously reported.[19] Cases from TMA slides with weak or negative internal control cases (tonsil tissue) after repeat staining were excluded from further analysis. The Ki67 proliferation index (PI) was automatically calculated by the software as a ratio (%) of positively stained nuclei to all nuclei. For each core a median of 3019 tumor nuclei were counted (range 19–12 091). Tumor PIs for each patient were averaged from three analyzed cores. The maximum score % Ki67 positivity per tumor was calculated as a surrogate of the 'hotspot' reflecting the area of most intense proliferation.[20,21] All cases were coded and analyzed by a pathologist in a blinded fashion, without any knowledge of patient outcome.

Statistical analysis

Patient characteristics were collected in the clinical data set and included pre-operative serum PSA level, pathology stage, GS, seminal vesicle invasion (SVI), extracapsular extension (ECE), and surgical margin status (positive or negative). Of 1326 patients with clinical data, 1004 patients had complete high-quality Ki67 tumor data available for analysis with acceptable strong uniform TMA staining of the positive controls.

The summary statistics of patient characteristics are provided in frequencies and percentages (see Table 1). Ki67 and pre-operative PSA data were summarized using mean, s.d. and range. The Wilcoxon rank sum test or Kruskal–Wallis test were used to compare Ki67 PI between patient groups. The Pearson correlation test was used to assess association between Ki67 PI and pre-operative PSA. Inter- and intra-tumor variation of Ki67-positive nuclei were estimated using variance component analysis. Ki67 were also dichotomized by either weighted average of tumor Ki67 or maximum percent positive nuclei using 5% cutoff point reported previously for RP/TURP cohorts as an independent prognosticator in multivariable analyses.[3,7,22–24]

The primary end points of this analysis were: (1) recurrence-free survival (RFS) post-surgery, where an event was defined as any PC recurrence (biological, clinical/radiological or treatment with salvage therapy) or metastasis or death due to PC; (2) overall survival (OS), where an event was defined as death of any cause; and (3) disease-specific survival (DSS), where an event was defined as PC metastasis or death due to PC. The baseline was set as the date of RP.

There were four groups of patients defined in this study: (1) non-recurrence (48% cases); (2) recurrence within 5 years of RP (40% cases); (3) lost-to-follow-up within 5 years of RP (6% cases); and (4) recurrence more than 5 years after RP (6% cases). The survival of patient groups was estimated using the Kaplan–Meier method and groups were compared using the log rank test. A univariable Cox model was used to assess the effects of Ki67 PI and other patient characteristics on RFS, OS and DSS. A backwards elimination procedure including PSA, GS, age, margin, ECE, SVI and Ki67 covariates was used to identify a final multivariable Cox model with all significant factors for each survival end point. The summaries of concordance index of different Cox proportional hazard models for RFS, types of recurrence and salvage therapy were analyzed.

All tests were two-sided and *P*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC, USA). Plots were produced using Spotfire S+ 8.2 (TIBCO, Palo Alto, CA, USA).

RESULTS

Correlating Ki67 proliferation index with clinicopathological characteristics

We determined the Ki67 PI in >4000 prostate tissue cores from 1004 RPs using the Aperio system for automated detection of staining and quantification of positive nuclei. The Ki67 PI ranged from 0 to 35.6% with weighted average median value of 2.19%. We found significant associations between Ki67 PI and pathologic stage, SVI, ECE, and GS (Table 1). More specifically, Ki67 PI was significantly higher in patients with pathologic stages pT3/pT4 vs stage pT2 ($P<0.0001$), as well as cases with SVI and

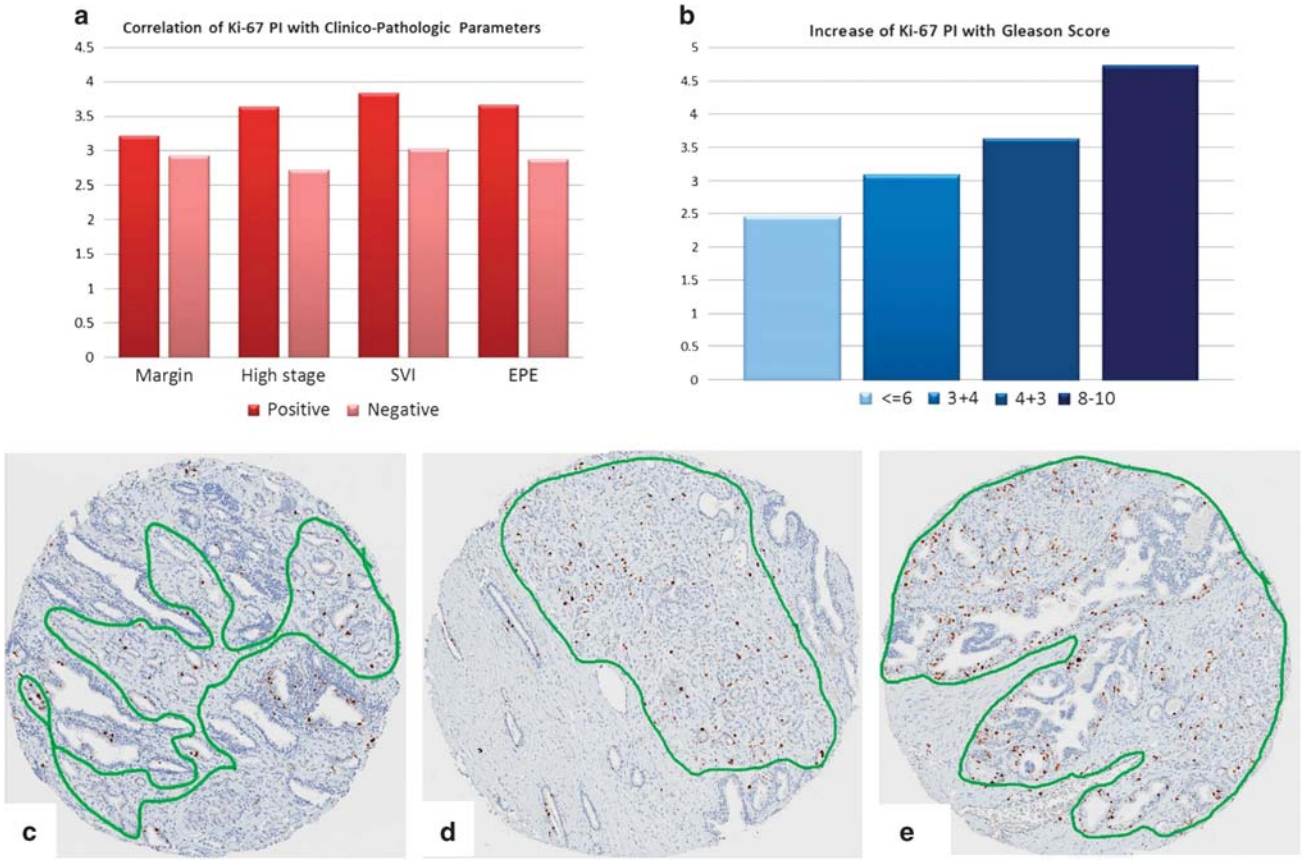

Figure 1. (**a**) Significant positive correlation is found between increased Ki67 PIs and higher pathology stage ($P < 0.0001$), seminal vesicle invasion (SVI; $P = 0.02$), extracapsular extension ($P < 0.0001$), but not with margin status ($P = 0.21$); (**b**) gradual increase of Gleason scores correlates with Ki67 PI ($P < 0.0001$); and (**c** and **d**) representative Ki67 immunostained tissue cores with Gleason scores 3+3 (**c**), 3+4 (**d**) and 4+3 (**e**) prostate cancers. The green line represents the areas circled manually for automated image analysis. PI, proliferation index.

ECE ($P = 0.02$ and $P < 0.0001$, respectively). There was also a statistically significant increase in Ki67 PI from lower to higher GS (Figure 1). Strong positive correlation between higher GS and higher Ki67 PIs were found for weighted average and maximum Ki67 PI by Kruskal–Wallis test ($P < 0.0001$). No significant association was found between Ki67 PI and positive surgical margin (PSM) status or pre-operative PSA by Wilcoxon rank sum test and Pearson's correlation ($P = 0.21$ and $P = 0.36$, respectively).

The intra-tumor variance of Ki67 expression, estimated from different tumor cores, was 0.39 (95% confidence interval: 0.36–0.41), whereas the inter-tumor variation was 0.68 (95% confidence interval: 0.61–0.76). The inter-tumor variation explained 64% of total variation.

Ki67 PI analysis as a continuous variable: univariable and multivariable analysis for RFS

The summary of 5-year RFS by Ki67 PI and clinicopathological factors are shown in Supplementary Table 1. The univariable Cox proportional hazard model determined that the weighted average Ki67 PI was significantly correlated with RFS as a continuous variable (per 1% increase, hazards ratio (HR) = 1.04, $P = 0.002$), as well as maximum % Ki67 positivity (per 1% increase, HR = 1.03, $P = 0.005$). Other PC characteristics significantly associated with RFS events were PSM, presence of SVI and ECE and (log) pre-operative PSA as continuous one unit incremental values ($P < 0.0001$). Age was not significantly associated with RFS either by itself or after being adjusted for Ki67, PSA, margin status, SVI and GS. Two multivariable Cox proportional hazard models (including 634 cases with 281 recurrences) for RFS demonstrated that higher weighted Ki67 PI average (model 1) and higher maximum Ki67% positivity (model 2) were both significantly correlated with worse RFS after adjusting for other clinical factors (PSM, SVI, GS, ECE and pre-operative PSA) ($P = 0.02$–0.0008; Table 2). The concordance index of different Cox proportional hazard models showed added value of Ki67 in predicting RFS post-surgery compared with only clinical data, more significant for continuous weighted average versus maximum Ki67 or dichotomized (Supplementary Table 2).

Ki67 PI analysis as a continuous variable: univariable and multivariable analysis for OS and DSS

The univariable Cox proportional hazard analysis demonstrated that significantly worse OS was associated with increasing Ki67 PI ($P = 0.003$) or pre-operative PSA ($P = 0.018$), stage pT3/pT4 vs pT2 ($P = 0.01$), presence of SVI ($P < 0.0001$) or ECE ($P = 0.01$) and GS of 8 or higher (vs 6 or lower) ($P = 0.008$). Worse DSS was also strongly associated with increasing Ki67 PI ($P = 0.004$) and pre-operative PSA ($P < 0.0001$), PSM ($P = 0.02$), stage pT3/pT4 vs pT2 ($P < 0.0001$), presence of SVI ($P = 0.01$) and GS of 6 vs 4+3 ($P < 0.0001$). The multivariable Cox proportional hazard model for OS comprised a total sample size of 984 with 57 events. After adjusting for pre-operative PSA, PSM, pathologic stage, SVI and ECE, worse OS was significantly associated with increasing by 1% Ki67 PI (HR = 1.09, $P = 0.02$) or GS of 8 or higher (vs 6 or lower) (HR = 3.28, $P = 0.0007$). The same model was run for DSS and comprised a total sample size of 874 with 44 events. There were

significant associations with worse DSS and increasing by 1% Ki67 PI (HR = 1.1, $P = 0.02$), pre-operative PSA (HR = 1.98, $P = 0.005$) and GS of 8 or higher (vs 6 or lower) (HR = 5.13, $P = 0.001$). Multivariable cox regression analysis results for RFS, OS and DSS are shown in Table 2.

Defining clinically useful cutpoints for Ki67 PI

We examined the functional form and possible cutoff points of Ki67 PI and pre-operative PSA using Martingale residual plots (Supplementary Figure 1). The linear form of Ki67 and logarithmic transformation of pre-operative PSA were used for modeling. Martingale residual plots showed no discrete cut-point that could be used to dichotomize samples prognostically using log(PSA) or KI67 PI values. Therefore, we tested three different Ki67 cutpoints to dichotomize cases to evaluate associations with clinical end points: weighted average (2.19%), maximum per case (3.11%), and at cut-point of 5% which has been used successfully in several previous studies of localized PC.[3,7,22–24]

The univariable Cox proportional hazard model showed significant correlation with RFS when dichotomized by median classes only for the weighted average (2.19%, HR = 0.72, $P = 0.01$), but not for the maximum % positive Ki67 per tumor (3.11%, HR = 0.86, $P = 0.06$). The multivariable Cox proportional hazard model for outcomes dichotomized by Ki67 PI weighted average and maximum Ki67% positivity were not significant. Moreover, no significant relationship with OS and DSS was detected with either Ki67 cutoff of 2.19% or 3.11%.

The multivariable Cox proportional hazard model showed significant correlation with RFS when Ki67 dichotomized at 5% cutoff for both the weighted average (model 1) and for the maximum % positive (model 2). Tumors with Ki67 $\geqslant$ 5% were associated with worse RFS after adjusting for pre-op PSA, margin status, SV invasion status, and Gleason score: HR = 1.47, $P = 0.0007$ (model 1) and HR = 1.31, $P = 0.03$ (model 2). The multivariable Cox proportional hazard model for OS comprised a total sample size of 992 with 57 events showed significant relationship with worse OS and Ki67 $\geqslant$ 5% based on weighted average (HR = 2.03, 95% confidence interval 1.09–3.8, $P = 0.03$). The same model was run for DSS and comprised a total sample size of 1135 with 51 events. Analysis of DSS demonstrated its significant relationship with Gleason scores (HR = 2.6–8.11, $P = 0.0004$) and (log) pre-operative PSA (HR = 2.01, $P = 0.006$), but not with Ki67 dichotomized at 5% cutoff. Multivariable cox regression analysis for RFS stratified by center is shown in Table 3.

Kaplan–Meier plots for all survival end points indicated that patients with tumor Ki67 PI above the median ($>$2.19%) had a significantly decreased probability of RFS ($P = 0.003$) (Figure 2a), which was even more evident with 5% cut-point ($P < 0.0001$) (Figure 2b). Moreover, Kaplan–Meier survival curves with 5% cutoff point showed a trend toward predicting DSS in tumors with higher Ki67 ($P = 0.083$) and significant difference for OS ($P = 0.045$), but not for cutoffs of 2.19% or 3.11%. All *P*-values in this paragraph were produced by the log-rank test.

Table 1. Summary of Ki67 by patient clinicopathological characteristics

Characteristics	*Weighted average Ki67 percent positive nuclei*						
	N	*Mean*	*s.d.*	*Min.*	*Median*	*Max.*	P-*value*
Margin							
Missing	167	3.31	3.16	0.07	2.53	23	
Negative	552	2.93	2.81	0	2.04	26.01	
Positive	285	3.22	3.08	0	2.23	25.5	0.21
Pathology stage							
Missing	193	3.4	3.42	0	2.38	23	
pT2	567	2.72	2.58	0	1.94	26.01	
pT3/pT4	244	3.64	3.25	0	2.79	25.5	$<$ 0.0001
Seminal vesicle invasion							
Missing	13	2.52	1.85	0.89	2.18	7.42	
No	925	3.03	2.95	0	2.14	26.01	0.02
Yes	66	3.84	3.11	0.21	3.41	15.62	
Extracapsular extension							
Missing	9	2.09	1.63	0.57	1.46	5.3	
No	742	2.88	2.78	0	2.02	26.01	$<$ 0.0001
Yes	253	3.67	3.38	0	2.69	25.5	
Gleason score							
Missing	8	3.27	4.44	0.56	1.36	13.63	
$\leqslant$6	400	2.46	2.1	0	1.93	14.68	$<$ 0.0001
3+4	370	3.09	2.59	0	2.42	23	
4+3	125	3.64	3.2	0.09	2.4	15.62	
8–10	101	4.74	5.12	0.22	2.77	26.01	
Total	1004	3.07	2.95	0	2.19	26.01	

P-values by Wilcoxon rank sum test or Kruskal–Wallis test, where appropriate.

DISCUSSION

Our Ki67 validation study was conducted in over 1000 radical prostatectomies in compliance with MISHFISHIE (minimum information specification for *in situ* hybridization and immunohistochemistry experiments)[25] and REMARK (Reporting recommendations for tumor marker prognostic studies).[26,27] Our objective was to evaluate whether Ki67 testing provides meaningful prognostication and whether an image-based automated scoring system could meet criteria for use in clinical practice. To our knowledge, this is the largest study to date, with one of the longest periods of follow-up (median: 7 years),[5,9,28] and represents a wide spectrum of patients with PC managed at six major centers across North America. We found that increased Ki67 as a continuous variable was significantly associated with stage pT3/pT4, presence of SVI or ECE, and higher GS, but not with PSM and/or pre-operative PSA levels. After adjusting for other factors on multivariable analysis, Ki67 as a continuous variable remained a significant independent predictor for recurrence-free, overall and disease-specific survival using logistic regression models and Cox proportional hazard models. These findings substantiate Ki67 staining as an independent predictive biomarker for PC outcomes.

Our tested Ki67% cutoffs of weighted average and maximum % positivity per case (hotspot equivalent) were 2.19% and 3.11%, respectively. These values were comparable to several other studies,[1,29,30] but at the lower end of broadly ranging cutoffs from 2.4% to 26%. The range of Ki67 PI values in these studies could reflect differences in the risk profiles of the patient cohorts, tumor heterogeneity, pre- and post-analytical variables, manual vs automated counting and different statistical methodologies in determining 'suitable cutpoints' as mean, median, maximal or quartile-based.[1,3,8,14,28,30,31] Our relatively low weighted average PI could also be partially explained by the study population that was selected to enrich for recurrent low-grade and non-recurrent high-grade cases, possibly leading to oversampling of cases with lower proliferative indices. However, our patient population reflects contemporary risk groups of PC diagnosed in North America, particularly patients who are candidates for active surveillance in whom prognostic biomarkers are most needed.

A recent study of PC patients treated on Radiation Therapy Oncology Group (RTOG 94-08) using automated ACIS scoring

Table 2. Ki67 analysis as a continuous variable: multivariable Cox proportional hazard models for RFS, OS and DSS

Model	*Factor*	*Comparison*	*Hazard ratio*	*95% LCL*	*95% UCL*	*Overall P-value*
RFS model 1 ($N=634$, #events = 281)	Ki67% weighted average	1% increase	1.07	1.03	1.11	0.0008
	Margin	Pos vs Neg	1.41	1.09	1.84	0.01
	Seminal vesicle invasion	Yes vs no	1.85	1.23	2.80	0.003
	Gleason score	3+4 vs 6	1.22	0.90	1.66	0.005
		4+3 vs 6	1.88	1.33	2.66	
		8–10 vs 6	1.42	0.96	2.09	
	Pathological stage	pT3/pT4 vs pT2	1.43	1.07	1.92	0.02
	Log(PSA)	One unit increase	1.62	1.35	1.96	<0.0001
RFS model 2 ($N=634$, #events = 281)	Ki67% maximum	1% increase	1.04	1.01	1.07	0.007
	Margin	Pos vs Neg	1.40	1.08	1.83	0.01
	Seminal vesicle invasion	Yes vs no	1.85	1.22	2.79	0.004
	Gleason score	3+4 vs 6	1.24	0.91	1.67	0.003
		4+3 vs 6	1.91	1.35	2.70	
		8–10 vs 6	1.46	0.99	2.15	
	Pathological stage	pT3/pT4 vs pT2	1.44	1.07	1.93	0.02
	Log(PSA)	One unit increase	1.61	1.34	1.95	$<.0001$
OS ($N=984$, #events = 57)	Ki67% positive	1% increase	1.09	1.01	1.16	0.02
	Gleason score	3+4 vs $\leqslant 6$	0.87	0.44	1.72	0.68
		4+3 vs $\leqslant 6$	1.14	0.46	2.84	0.78
		8–10 vs $\leqslant 6$	3.28	1.65	6.51	0.0007
DSS ($N=874$, #events = 44)	Ki67% positive	1% increase	1.10	1.02	1.18	0.02
	Log (PSA)	One unit increase	1.98	1.35	2.89	0.005
	Gleason Score	3+4 vs $\leqslant 6$	2.27	0.93	5.52	0.07
		4+3 vs $\leqslant 6$	2.75	0.97	7.81	0.06
		8–10 vs $\leqslant 6$	5.13	1.92	13.75	0.001

Abbreviations: DSS, disease-free survival; LCL, lower confidence level; Neg, negative; OS, overall survival; PC, prostate cancer; PI, proliferation index; Pos, positive; RFS, recurrence-free survival; UCL, upper confidence level. RFS event is defined as any recurrence, metastasis or PC death. Models 1 and 2 are separate models using Ki67 weighted average % positive (PI = 2.19) and maximum % positive (PI = 3.11), respectively. OS event is defined as death of any cause. DSS event is defined as PC metastasis or death due to PC. Both OS and DSS have only one final model. Only significant factors were included in the final models.

Table 3. Ki67 dichotomized at 5% cutoff: multivariable Cox proportional hazard models stratified by center for RFS

Model	*Factor*	*Comparison*	*Hazard ratio*	*95% LCL*	*95% UCL*	*Overall P-value*
RFS model 1 ($N=706$, #events = 319)	Ki67% weighted average	$\geqslant 5\%$ vs $<5\%$	1.471	1.110	1.950	0.0007
	Margin	Pos vs Neg	1.598	1.248	2.047	0.0002
	Seminal vesicle invasion	Yes vs No	2.072	1.430	3.002	0.0001
	Gleason score	3+4 vs 6	1.297	0.972	1.732	0.004
		4+3 vs 6	1.808	1.273	2.568	
		8–10 vs 6	1.626	1.126	2.348	
	Log(PSA)	One unit increase	1.536	1.288	1.831	<0.0001
RFS model 2 ($N=706$, #events = 319)	Ki67% maximum	$\geqslant 5\%$ vs $<5\%$	1.314	1.033	1.672	0.03
	Margin	Pos vs Neg	1.596	1.246	2.043	0.0002
	Seminal vesicle invasion	Yes vs No	2.119	1.462	3.070	<0.0001
	Gleason score	3+4 vs 6	1.317	0.986	1.757	0.0002
		4+3 vs 6	1.876	1.324	2.658	
		8–10 vs 6	1.670	1.159	2.406	
	Log(PSA)	One unit increase	1.505	1.264	1.792	<0.0001

Abbreviations: LCL, lower confidence level; Neg, negative; PI, proliferation index; Pos, positive; RFS, recurrence-free survival; UCL, upper confidence level. RFS event is defined as any recurrence, metastasis, or prostate cancer death. Models 1 and 2 are separate models using Ki67 weighted average % positive (PI = 2.19) and maximum % positive (PI = 3.11), respectively. Only significant factors were included in the final models.

showed median Ki67 PI similar to what we observed (2.65%), although the automated scoring was slightly lower than the median value obtained by manual scoring in the same cohort (3.85%).[30] Manual scoring likely produces higher PIs because there typically are smaller numbers of nuclei counted and regions with more stained nuclei (hotspots) are likely to be oversampled. In agreement with these observations, median Ki67 PI in our study was nearly identical to RTOG 94-08 (PI = 2.19%) while the maximum Ki67 PI% positivity per case in our study (PI = 3.11%) was remarkably similar to manually scored hotspots.

Implementation of automated scoring in our high-throughput study was justified since (1) it provides more accurate

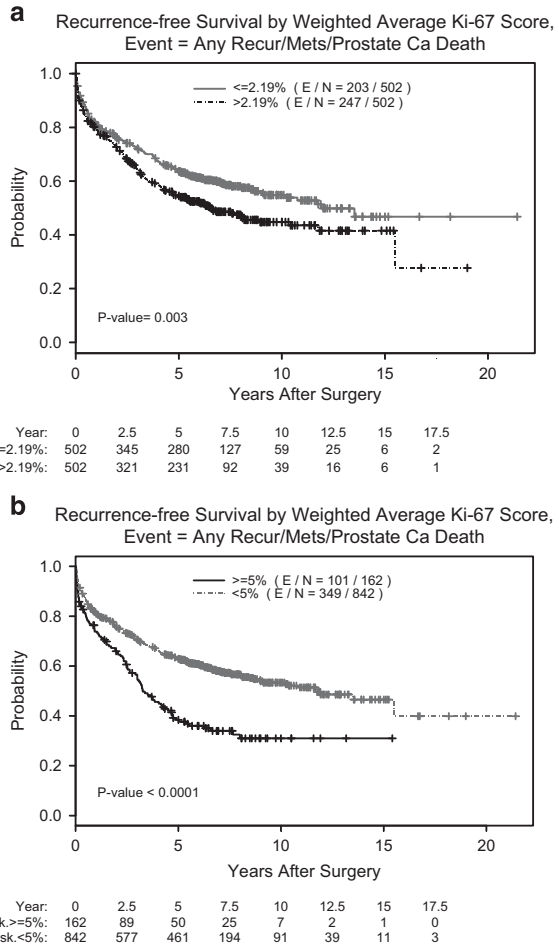

Figure 2. (**a**) Kaplan–Meier plot for recurrence-free survival using median as a cut-point. Cases with higher Ki67 proliferation index (>2.19%, blue dotted line) have significantly decreased probability of recurrence-free survival ($P = 0.003$) when compared with patients with Ki67 proliferation index equal or below median (black solid line); (**b**) Kaplan–Meier plot using Ki67 proliferation index of 5% as a cut-point. Cases with Ki67 proliferation index >5% (black solid line) have significantly decreased probability of recurrence-free survival ($P < 0.0001$) when compared with patients with Ki67 proliferation index ⩽5% (blue dotted line).

determination of Ki67 PI because higher numbers of counted cells (a median of 3019 tumor nuclei), (2) it reduces human error and fatigue during quantitating of ~4000 cores and (3) it eliminates scoring variations by analysis of uniformly batch stained TMA slides with a standard nuclear counting algorithm. Furthermore, the accuracy of automated scoring has been separately validated in studies, in which adequate sample sizes for manually counted nuclei were obtained. A comparative study between digital image analysis using the same Aperio XT nuclear algorithm and manual counting of adequate numbers of cells (>2,000 nuclei) showed excellent concordance with interclass correlation of 0.98.[19]

Our data suggest two alternate approaches for incorporating Ki67 PI into clinical practice. In multivariable analysis, both median and maximum KI67 PI provided independent prediction of RFS when coupled with other clinical and pathological parameters. In this case, KI67 PI could be incorporated into clinical risk models, such as CAPRA or other nomograms, although appropriate weighting of the models might need to be adjusted for a non-selected population. Alternatively, biomarkers with a purely dichotomous output (positive or negative) that sort patients into high- and low-risk groups are easier to incorporate into risk models for clinical use.[14,31] Despite our inability to define a cut-point of Ki67 PI in our data set using the Lowess smoothed plot of Martingate residuals, we did validate that a cut-point of 5%, which had been derived empirically in previous studies,[3,7,22–24] did provide independent prediction of RFS with a HR (1.47) comparable to Gleason score (HR = 1.29–1.81), margin status (HR = 1.59) and log(PSA) increase by one unit (HR = 1.54), although slightly lower than SVI (HR = 2.07). In addition, Kaplan–Meier plots showed robust curve separation between low and high Ki67 groups at 5% that outperformed median or maximum Ki67 PI per case. Whether 5% is the ideal cut-point is unclear, given the results of our cut-point analysis. However, Kaplan–Meier analysis also showed that tumors with very low Ki67 PI (⩽1%) were indistinguishable from those that were 1–5%, implying that the cut-point is likely to be at 5% or greater.

Our study has some limitations, since it is retrospective and based on radical prostatectomy samples and was designed with specific parameters to help in identifying prognostic biomarkers that are independent of clinical predictors. For example, the selection of balanced numbers of recurrent and non-recurrent Gleason score 3+4 and 4+3 cases and oversampling of recurrent 3+3 and non-recurrent 4+4 cases improved our ability to validate markers that predict outcomes after surgery that are independent of known clinical predictors such as Gleason score. Because of this, the relative weight of the biomarker in predicting outcome cannot be incorporated into existing algorithms, such as CAPRA, that have been developed and tested in non-selected surgical populations. In addition, selection of the region of highest grade in construction of the TMA could limit application of the findings to non-selected random biopsies and further confounded by intratumoral heterogeneity. In addition, there is considerable overlap in Ki67 PI in univariable analysis between favorable and high-risk clinical and pathological outcomes, making clinical translation challenging. Very likely, the greatest utility will be derived from combining Ki67 PI with independent predictors, or by focusing more on extreme values, such as the >5% cut-point. Translation to clinically relevant scenarios, such as selection of patients for active surveillance in the low-risk population or for adjuvant therapies in high-risk localized disease will require testing in prospective cohorts. While our study is based on small samples of cancer, in many ways comparable to standard prostate needle biopsies, it will need to be validated on biopsy samples because of potential issues with sampling error in prostate needle biopsies. It is possible that the advent of image-directed biopsies using multiparametric magnetic resonance imaging could significantly improve the performance of prognostic biomarkers because of improved sampling the largest incident lesion. However, this hypothesis needs to be tested.

CONCLUSIONS

In localized PC treated by radical prostatectomy, Ki67 PI provides independent prognostic value for RFS, DSS and OS beyond Gleason score, pathological stage and PSA levels. In our large, multi-institutional cohort, Ki67 PI performed best as a continuous variable and could be incorporated into existing or new predictive nomograms. In addition, our study suggests that risk stratification for localized PC could be achieved with Ki67 as a dichotomous variable at 5% cutoff. These findings strongly suggest that Ki67 PI should be further tested as a prognostic biomarker in other clinically relevant cohorts such as patients on active surveillance,[32] and possibly in patients undergoing image guided biopsies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Analytic Validation of a Clinical-Grade PTEN Immunohistochemistry Assay in Prostate Cancer by Comparison to *PTEN* FISH

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Abstract

PTEN loss is a promising prognostic and predictive biomarker in prostate cancer. Because it occurs most commonly via *PTEN* gene deletion, we developed a clinical-grade, automated and inexpensive immunohistochemical assay to detect PTEN loss. We studied the sensitivity and specificity of PTEN immunohistochemistry relative to 4-color fluorescence *in situ* hybridization (FISH) for detection of *PTEN* gene deletion in a multi-institutional cohort of 731 primary prostate tumors. Intact PTEN immunostaining was 91% specific for absence of *PTEN* gene deletion, (549/602 tumors with 2 copies of the *PTEN* gene by FISH showed intact expression of PTEN by immunohistochemistry) and 97% sensitive for presence of homozygous *PTEN* gene deletion (absent PTEN protein expression by immunohistochemistry in 65/67 tumors with homozygous deletion). PTEN immunohistochemistry was 65% sensitive for presence of hemizygous *PTEN* gene deletion, with protein loss in 40/62 hemizygous tumors. We reviewed the 53 cases where immunohistochemistry showed PTEN protein loss and FISH showed 2 intact copies of the *PTEN* gene. On re-review, there was ambiguous immunohistochemistry loss in 6% (3/53) and failure to analyze the same tumor area by both methods in 34% (18/53). Of the remaining discordant cases, 41% (13/32) revealed hemizygous (n=8) or homozygous (n=5) *PTEN* gene deletion that was focal in most cases (11/13). The remaining 19 cases had 2 copies of the *PTEN* gene by FISH, representing truly discordant cases. Our automated PTEN immunohistochemistry assay is a sensitive method for detection of homozygous *PTEN* gene deletions. Immunohistochemistry screening is particularly useful to identify cases with heterogeneous *PTEN* gene deletion in a subset of tumor glands. Mutations, small insertions or deletions and/or epigenetic or microRNA-mediated mechanisms may lead to PTEN protein loss in tumors with normal or hemizygous *PTEN* gene copy number.

Keywords

Prostatic carcinoma PTEN; fluorescence in situ hybridization; immunohistochemistry; radical prostatectomy; biomarker

Introduction

PTEN is the most commonly lost tumor suppressor gene in prostate cancer (1-5) and is a promising prognostic biomarker for poor clinical outcomes (6-18). Since the *PTEN* gene is almost always lost by genomic deletion of the entire gene in prostate tumors, fluorescence *in situ* hybridization (FISH) has traditionally been the gold standard assay to detect *in situ PTEN* loss in tumor tissue. However the relatively recent availability of reliable rabbit monoclonal antibodies for detection of PTEN protein has enabled the development of highly validated immunohistochemistry protocols to detect PTEN loss in prostate cancer (9, 19). Immunohistochemistry-based detection of PTEN loss in prostate cancer is less expensive and less time-consuming than FISH for the routine screening of prostate tumor specimens, making it easier to adapt to the current pathology work flow for risk assessment in prostate cancer. In addition, since *PTEN* loss is commonly subclonal and heterogeneous in primary prostate tumors (9, 20-22), detection of *PTEN* gene deletion by FISH can be technically challenging in some cases and screening for focal loss may be more easily accomplished by immunohistochemistry. Finally, there is emerging evidence that in addition to genetic

deletion, PTEN protein levels may be compromised by mutations in the gene or microRNA- or epigenetic-regulated mechanisms which would not be detectable by FISH (9, 23-25).

We previously optimized and validated a PTEN immunohistochemistry assay for the detection of PTEN loss in prostate cancer specimens (9), and PTEN loss by this assay correlated with increased risk of biochemical recurrence in a case-control cohort of patients undergoing radical prostatectomy (12) and with risk of progression and metastasis in two high risk surgical cohorts (though the latter was not significant in multivariate analyses) (9, 11). Though originally performed manually, we have recently transferred this assay to a clinical-grade automated immunostaining platform that may be run in any CLIA-certified pathology laboratory. Using this assay, we recently reported that PTEN loss is associated with reduced recurrence free survival in multivariable models in a multi-institutional cohort of surgically treated patients (26) and with higher risk of lethal prostate cancer in a large population-based cohort (18). *PTEN* gene deletion by FISH has also been recently reported in a subset of the multi-institutional cohort and correlated with recurrence free survival (17). Here, to analytically validate our clinical-grade PTEN immunohistochemistry assay, we compared the performance of the automated immunohistochemistry assay to *PTEN* FISH in this cohort, one of the largest multi-institutional cohorts to be studied by both techniques. We demonstrate that our immunohistochemistry assay shows robust sensitivity and specificity for detection of homozygous *PTEN* gene deletion.

Methods

Subject selection and tissue microarray design

The Canary Foundation Retrospective Prostate Tissue Microarray Resource has been described in detail elsewhere (27). Briefly, it is a multicenter, retrospective prostate cancer tissue microarray created as a collaborative effort with radical prostatectomy tissue from six academic medical centers: Stanford University, University of California San Francisco, University of British Columbia, University of Washington (including tissues from University of Washington and a separate cohort from the Fred Hutchinson Cancer Research Center), University of Texas Health Science Center at San Antonio, and Eastern Virginia Medical School. Tumor tissue from 1275 patients was selected for the tissue microarray using a quota sampling plan, from radical prostatectomy specimens collected between 1995 and 2004. A starting date of 1995 was selected to enrich for cases occurring after the implementation of PSA screening. There was no central pathology review in this cohort. The tissue microarray included samples from men with (a) recurrent prostate cancer; (b) nonrecurrent prostate cancer; and (c) unknown outcome due to inadequate follow-up time (ie, censoring). Recurrent cases of Gleason score 3+3=6 and 3+4=7 were relatively over-sampled as well as non-recurrent cases with Gleason score 4+4=8, in order to improve power to detect biomarkers providing prognostic information independent of Gleason score.

Each site built 5 tissue microarrays, each containing tumor tissue from 42 patients (210 patients from each contributing site). Each tumor was sampled in triplicate, utilizing 1 mm cores and an additional core of histologically benign peripheral zone tissue was included for each patient as a control. Recurrent and non-recurrent patients were distributed randomly across all tissue microarrays.

Immunohistochemistry assays

PTEN immunohistochemistry was performed on the CFRPTMR cohort as recently reported (26). Briefly, the protocol uses the Ventana automated staining platform (Ventana Discovery Ultra, Ventana Medical Systems, Tucson, AZ) and a rabbit anti-human PTEN antibody (Clone D4.3 XP; Cell Signaling Technologies, Danvers, MA). We previously validated a manual version of this assay using the same antibody in genetically characterized cell lines and prostate tumor tissue, showing strong correlation of the immunohistochemistry with *PTEN* gene copy number by 2-color FISH and high resolution SNP array analysis (9) and good correlation with 4-color FISH in a small cohort of needle biopsy specimens (28). To prove equivalence between the manual and automated assays, we also examined a test tissue microarray containing 50 prostate cancer cases with known PTEN protein status (including more than 30 with PTEN protein loss) by manual staining and found 100% concordance between the PTEN protein status on the manual and automated platforms.

Immunohistochemistry scoring

After staining for PTEN, all tissue microarrays were scanned at 20× magnification (Aperio, Leica Microsystems, Buffalo Grove, IL) and segmented into TMAJ for scoring (http://tmaj.pathology.jhmi.edu/). PTEN protein status was blindly and independently scored by two trained pathologists (TLL and CLM) using a previously validated scoring system (see below). Overall, there was "very good" agreement between independent reviewers, with 96% agreement over 2783 cores scored by both reviewers ($\kappa = 0.905$; 95% CI=0.887-0.923) (26).

A tissue core was considered to have PTEN protein loss if the intensity of cytoplasmic and nuclear staining was markedly decreased or entirely negative across >10% of tumor cells compared to surrounding benign glands and/or stroma, which provide internal positive controls for PTEN protein expression (9). If the tumor core showed PTEN protein expressed in >90% of sampled tumor glands, the tumor was scored as PTEN intact. If PTEN was lost in <100% of the tumor cells sampled in a given core, the core was annotated as showing heterogeneous PTEN loss in some, but not all, cancer glands (focal loss). Alternatively, if the core showed PTEN loss in 100% of sampled tumor glands, the core was annotated as showing homogeneous PTEN loss. Finally, a small percentage of cores were scored as having ambiguous PTEN immunohistochemistry results. This occurred when the intensity of the tumor cell staining was light or absent in the absence of evaluable internal benign glands or stromal staining. The percent of tissue cores with ambiguous scoring for PTEN immunohistochemistry was fairly constant across 6 of the 7 institutions included in the Canary tissue microarray cohort and varied from 0.7%-5.3% (26).

For statistical analysis, each patient's tumor sample was scored for the presence or absence of PTEN loss by summarizing the scores of each individual sampled core from that tumor. A patient's tumor was designated as having heterogeneous PTEN loss if at least one tumor core showed heterogeneous PTEN loss, or alternatively, if at least one core showed heterogeneous or homogeneous PTEN loss and at least one core showed PTEN intact in tumor cells. A patient's tumor was scored as showing homogeneous PTEN loss if all sampled tumor cores showed homogeneous PTEN loss. Finally, a patient's tumor was scored

as having PTEN intact if all sample tumor cores showed intact PTEN in sampled tumor glands.

Initial blinded analysis of *PTEN* FISH

PTEN FISH was performed as previously described for a subset of this cohort (17). Briefly, the PTEN Del TECT FISH utilizes a four color probe combination as described. Probes were supplied by CymoGenDx LLC (New Windsor, NY) as follows: centromeric copy control probe - CYMO-Pink; *WAPAL* – CYMO-Green; *PTEN* – CYMO-Red; and *FAS* – CYMO-Aqua. We have shown previously that use of the probes bracketing *PTEN* improves the fidelity of assessments of PTEN loss (29). The two probes *WAPAL* and *FAS* on either side of PTEN provide information about the size of larger deletions and also allow recognition of background artifactual losses of *PTEN* due to histologic sectioning. Artifacts in assessing *PTEN* loss can arise when histologic sectioning cuts away part of the nucleus containing the *PTEN* locus in cells in the section while leaving the centromere in place. The latter is a result of the long distance between the centromere and the *PTEN* locus on chromosome 10.

PTEN FISH analysis was performed entirely independently of PTEN immunohistochemistry, using 5 micron tissue microarray sections stained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) in tumor areas selected by a pathologist who was not involved in PTEN immunohistochemistry scoring (TJ) using an immediately adjacent section stained with hematoxylin and eosin. *PTEN* copy number was evaluated by counting spots for all four probes using SemRock filters appropriate for the excitation and emission spectra of each dye in 50–100 non-overlapping, intact, interphase nuclei per tumor tissue microarray core. For the initial blinded analysis of each case, two tumor-containing cores were scored based on the overall quality of FISH hybridization. In cases where different clonal deletions were present, all three cores were analyzed and more cells were analyzed. Hemizygous (single copy) *PTEN* loss was assigned when >50% of nuclei exhibited either interstitial loss of *PTEN* or concomitant loss of adjacent genes (*PTEN* and *WAPAL* and/or *FAS*). Homozygous deletion was defined by a simultaneous lack of both *PTEN* locus signals in 30% of scored nuclei.

Immunohistochemistry-guided re-analysis of cases with discrepant results by immunohistochemistry and FISH

53 cases showed PTEN protein loss by immunohistochemistry with 2 copies of *PTEN* gene present by initial FISH analysis (see Results, below). Two cases showed PTEN protein intact by immunohistochemistry with homozygous *PTEN* deletion by *PTEN* FISH. To analyze the cause of these discrepancies, we re-examined both the immunohistochemistry and FISH data in these cases. A digitally scanned photomicrograph of the most representative core with immunohistochemistry loss was selected to guide FISH re-analysis of the identical core from each case. Since the majority (85%) of these discrepant cases showed only focal immunohistochemistry loss in a subset of glands, the FISH re-analyses concentrated on determining the *PTEN* gene copy number within these small areas guided by the immunohistochemistry staining. Since only 50-100 cells from the best two of the three tumor-containing cores were initially analyzed for each case by *PTEN* FISH (29), this more

extensive analysis could include tissue microarray cores and regions of tissue microarray sections that had not been studied by FISH during initial blinded analysis.

Immunohistochemistry and FISH on standard tissue sections

To examine possible effects of tumor heterogeneity on immunohistochemistry and FISH interpretation in the setting of tissue microarray cores, we additionally examined 20 cases of varying PTEN status (enriched for discordance between immunohistochemistry and FISH) by FISH and immunohistochemistry on standard tissue sections. Immunohistochemistry and FISH interpretation of these sections was performed blinded to the results of the tissue microarray analysis and the results of the other methodology.

Results

Data for PTEN FISH and immunohistochemistry in a subset of the CFRPTMR cohort were separately reported previously (17, 26). Briefly, of the 1275 patients with tissue sampled for the tissue microarrays, 86% (1095/1275) had evaluable PTEN status by immunohistochemistry and 14% (180/1275) had missing data (Supplementary Table S1). Of these, 17% (30/180) were missing due to ambiguous immunostaining results and 83% (150/180) had absence of tumor tissue present on the tissue microarray slides. Of the tumors with evaluable staining, 24% (258/1095) showed any PTEN protein loss, with 14% (150/1095) showing heterogeneous PTEN loss (in some but not all sampled tumor glands, best exemplified by case #10 in Figure 4A), and 10% (108/1095) showing homogeneous PTEN loss (in all sampled tumor glands). The remaining 76% (837/1095) of cases had intact PTEN protein by immunohistochemistry in all sampled tumor glands. *PTEN* FISH results were evaluable in 64% of the cases sampled on the tissue microarray (810/1275). Of the evaluable cases, *PTEN* FISH showed any *PTEN* deletion in 18% of cases, with 9% (70/810) of cases showing hemizygous deletion and 9% (75/810) of cases showing homozygous *PTEN* deletion. The remaining 82% (665/810) of cases showed two intact *PTEN* alleles.

PTEN immunohistochemistry results were available on 90% of cases with interpretable *PTEN* FISH results (731/810). The rates of *PTEN* gene and PTEN protein loss were quite similar in the subset with both FISH and immunohistochemistry results compared to the entire evaluable cohort for each assay reported separately. Overall, 22% (158/731) of cases with interpretable immunohistochemistry and FISH results showed PTEN protein loss, with 13% (96/731) showing heterogeneous loss and 8% (62/731) showing homogeneous loss. Similarly, 17% (129/731) of cases with interpretable immunohistochemistry and FISH results showed *PTEN* gene deletion (8% hemizygous and 9% homozygous).

Overall, intact PTEN immunohistochemistry was 91% specific for lack of underlying *PTEN* gene deletion. Of cases with 2 copies of the *PTEN* gene by FISH analysis, 549/602 showed intact PTEN protein (Figure 1, Tables 1 and 2). Notably, 85% (45/53) of the discrepant cases (loss of PTEN protein expression by immunohistochemistry and 2 copies of *PTEN* gene by FISH analysis) showed heterogeneous PTEN protein loss in some, but not all, sampled tumor glands, suggesting the possibility that a small area with *PTEN* deletion may have been missed in the initial FISH analysis (see below). PTEN immunohistochemistry loss was 65% sensitive for the detection of underlying hemizygous *PTEN* gene deletion since 40/62 of

cases with hemizygous *PTEN* gene deletion by FISH showed PTEN protein loss by immunohistochemistry (Figure 2). Of these cases, 65% (26/40) showed heterogeneous PTEN loss in some but not all sampled tumor glands. PTEN immunohistochemistry loss was 97% sensitive for homozygous *PTEN* gene deletion. Of cases with homozygous gene deletion by FISH, 65/67 showed PTEN protein loss by immunohistochemistry (Figure 3). Only 37% (25/67) of the cases with homozygous *PTEN* gene deletion and PTEN protein loss had heterogeneous loss of PTEN protein by immunohistochemistry. The fraction of tumors with underlying homozygous *PTEN* gene deletion differed by the extent of PTEN protein loss observed: 26% (25/96) tumors with heterogeneous PTEN protein loss had an underlying homozygous *PTEN* deletion compared to 64% (40/62) of tumors with homogeneous PTEN protein loss ($p<0.0001$ by Fisher's exact test).

The negative predictive value for intact PTEN immunohistochemistry was 96% (549/573) for lack of any gene deletion and 99.6% (571/573) for lack of homozygous *PTEN* deletion (Table 2). The positive predictive value of PTEN immunohistochemistry loss for presence of any *PTEN* gene deletion (homozygous or hemizygous) was 66% (105/158) overall, or 53% (51/96) for heterogeneous PTEN protein loss and 87% (54/62) for homogeneous PTEN protein loss (Table 2).

Next, we re-examined cases where there was a discrepancy between the PTEN immunohistochemistry and FISH. Overall, 53 cases with PTEN protein loss had two intact copies of *PTEN* by FISH, of which 85% (45/53) showed heterogeneous PTEN protein loss. Since only 50-100 tumor cells from two of the three tumor cores from each cases were initially evaluated by FISH, it is possible that focal tumor areas with *PTEN* gene deletion by FISH were missed or not analyzed in this blinded analysis. To examine this and other possible explanations for the immunohistochemistry-FISH discrepancy, each of these 53 discordant cases were re-reviewed for immunohistochemistry and FISH staining. Immunohistochemistry-guided FISH re-analysis in these cases revealed borderline immunohistochemistry loss in 6% (3/53) cases (Figure 4A, Case #10) and failure to analyze the identical tumor core or area by both immunohistochemistry and FISH in 34% (18/53) cases. Of the remaining discrepant cases where the immunohistochemistry result was convincing and the identical tumor area was analyzed by both methods, 41% (13/32) revealed hemizygous (n=8, Figure 4A, Case #11) or homozygous (n=5, Figure 4A, Case #12) deletion that was focal in 94% (11/13) cases and thus likely missed on initial FISH analysis. The remaining 59% (19/32) of these cases showed two copies of *PTEN*, thus representing truly discordant cases. One explanation for these cases is presence of a small deletion and/or mutation undetectable by FISH at one or both *PTEN* alleles. Another possibility is that even though the same core was evaluated by both methods in these cases, there may be heterogeneity within the core such that different levels of the core sampled on the FISH and immunohistochemistry slide may have been truly heterogeneous (Figure 4B, Case # 13). Of the two discrepant cases with homozygous *PTEN* deletion and intact PTEN protein, different tumor areas were analyzed in one case. In the other case, a minute focus of tumor with PTEN loss by immunohistochemistry that was initially missed was observed on re-examination (Figure 4B, Case # 14).

Finally, to further assess the effects of tumor heterogeneity on PTEN immunohistochemistry and FISH results and to determine whether this might account for discordance in some cases, we blindly studied a subset of 20 cases from the tissue microarray using standard tissue sections and compared results of immunohistochemistry and FISH on standard sections to one another and to those obtained for the tissue microarray cores of the same cases (Table 3). Cases chosen for this analysis were relatively enriched for discordance between tissue microarray-based immunohistochemistry and FISH results. In cases where the immunohistochemistry and FISH were concordant on the tissue microarray cores, results were generally highly concordant using standard sections as well. For example, in 3 cases where there was heterogeneous PTEN loss by immunohistochemistry and homozygous *PTEN* l*oss* by FISH in the tissue microarray cores, two of these tumors had clonal homozygous *PTEN* deletions, and the third tumor had a region with homozygous loss surrounded by a larger area with *PTEN* hemizygous loss. Similarly, in 4 cases that were PTEN intact by both immunohistochemistry and FISH on tissue microarray cores, 3 showed *PTEN* intact by FISH on standard sections (the fourth case failed to hybridize) and 3 showed PTEN intact by immunohistochemistry on standard sections (the fourth case showed focal PTEN loss). In some cases where there was discordance between the immunohistochemistry and FISH results on tissue microarray cores, more detailed analysis of standard sections suggested that tumor heterogeneity may be the underlying cause. Of 8 cases with heterogeneous PTEN loss by immunohistochemistry and intact *PTEN* by FISH on tissue microarray, 4/8 showed either hemizygous or homozygous *PTEN* loss by FISH on standard sections. Another case with homogeneous PTEN loss by immunohistochemistry and intact *PTEN* by FISH on tissue microarray revealed hemizygous *PTEN* loss by FISH on analysis of standard sections. Overall, these results support the possibility that underlying tumor heterogeneity is one potential cause of PTEN immunohistochemistry-FISH discordance. Despite this, tissue microarray-based evaluation of tumor PTEN status appears to be highly concordant with standard section analysis in most cases.

Discussion

There is an increasing need for validated prognostic and predictive biomarkers in prostate cancer at both ends of the clinical spectrum. Developing prognostic biomarkers to help select patients who are appropriate for active surveillance, as well as predictive biomarkers to guide the application of targeted therapy in metastatic disease remain major areas of unmet clinical need. PTEN has long been a promising marker in both regards, however, until relatively recently the lack of well validated antibodies to detect PTEN loss has made it challenging to incorporate into routine pathologic risk assessment protocols or clinical trials of PI3K-targeted agents in prostate cancer. Due to this difficulty, FISH has historically been used to assess whether PTEN is an effective prognostic biomarker by testing the association of *PTEN* gene deletion with prostate cancer progression. The results from these studies have consistently shown that *PTEN* gene deletion is associated with increased Gleason grade and stage in prostate cancer (6, 8, 10, 17, 30, 31). In addition, *PTEN* gene deletion is associated with prostate cancer progression and death in multivariable models (6-16). Though many of these previous studies have used 2-color FISH, there is increasing evidence that 4-color probes are better suited to distinguish true gene deletions from sectioning artifacts in

interphase FISH studies (Yoshimoto et al in preparation). Accordingly, our group recently demonstrated that homozygous PTEN deletion by 4-color FISH is associated with decreased recurrence-free survival in a subset of the prostate tumor cohort examined in the current study (17).

Despite these compelling data, *PTEN* FISH has not been widely implemented in clinical prostate cancer risk stratification protocols to date for a number of reasons. First, FISH to detect gene deletions is technically challenging, requiring careful probe design (29) and rigorous cutoffs to ensure that sectioning artifacts do not result in false calls of deletion. Detecting of hemizygous deletions can be particularly challenging when nuclei are overlapping or have been distorted during preparation. Depending on tissue quality and fixation, there may also be difficulties with optimizing protease digestion such that as many as 30-40% of cases cannot be evaluated on the first attempt when using tissue microarrays, though this may be less of an issue for biopsies (17). In large part because it is so technically challenging, FISH is relatively expensive compared to immunohistochemistry, and it has been harder to integrate the daily workflow of pathology laboratories as a reflexive test. Finally, though *PTEN* is most commonly lost via larger genomic deletions in prostate cancer, as many as 10-20% of cases may have mutations, small insertions or deletions that are not detectable by FISH, in addition to potential epigenetic and miRNA-mediated mechanisms of PTEN loss (1-5, 32). To address these challenges, several groups have developed immunohistochemistry assays to query PTEN status in tissue (9, 19, 33). While a number of such assays have been published, for the most part, these assays have largely been compared to 2-color FISH in only small scale studies with around 100 tumors each (23, 24, 34, 35). In the only large studies to compare immunohistochemistry and FISH, there was only weak (κ=0.5) (14) or no significant correlation (13) between the assays, suggesting a failure of the immunohistochemistry and/or FISH assay to analytically validate.

We used a commercially available rabbit monoclonal antibody to develop an immunohistochemistry assay to assess PTEN protein loss in prostate cancer and showed that this assay is reasonably sensitive for detection of *PTEN* gene deletion by 2-color FISH or high density SNP array in prostate cancer samples and shows minimal inter-observer variability in interpretation (9). Similarly, the assay performed well versus 4-color FISH in a small cohort of needle biopsy specimens (28). Using this assay, our group previously demonstrated that PTEN protein loss is associated with an increased risk of recurrence and progression in surgically treated cohorts of prostate cancer patients (11, 12).

To facilitate clinical use of the assay, we adapted it to the automated Ventana staining platform with clinical-grade reagents suitable for *in vitro* diagnostic use. This assay was clinically validated in a recent study showing that PTEN loss is associated with increased risk of lethal prostate cancer in a large population-based cohort in multivariable models (18). Despite a 4-category scoring system, the assay has shown high inter-observer reproducibility in a number of cohorts (including the current one), with κ values exceeding 0.9 (18, 26). In the current study, we analytically validated this automated assay by comparing it to 4-color *PTEN* FISH across a large multi-institutional cohort of prostate cancer patients. Remarkably, we found that the automated immunohistochemistry assay was 91% specific for 2 intact copies of the *PTEN* gene and 97% sensitive for homozygous *PTEN* gene deletions. This is

by far the highest sensitivity and specificity reported for a PTEN immunohistochemistry assay relative to FISH. This improved sensitivity and specificity is in part due to the improved specificity of the automated immunohistochemistry assay versus the manual assay and also due to the improved 4-color FISH assay which uses two *PTEN* gene flanking probes, in addition to a centromeric control and a *PTEN* probe to detect *PTEN* gene deletions. Surprisingly, the immunohistochemistry assay was also 65% sensitive for detection of hemizygous *PTEN* gene deletion, suggesting that there is complete protein loss in a large fraction, perhaps even a majority, of apparently hemizygous cases. This is most likely due to truncating mutations (nonsense, frameshift and splice site mutations) or epigenetic modifications at the second allele that are undetectable by FISH yet lead to protein loss (1, 3, 5, 36). Interestingly, though the prevalence of such mutations in *PTEN* is below 5% in most prostate tumors, many of these mutations are truncating alterations occurring in cases with hemizygous deletions that would lead to protein loss detectable by immunohistochemistry (1-5).

In addition to the potential increased sensitivity of immunohistochemistry versus FISH for detecting combinations of events including copy loss, point mutations, small insertions and deletions and epigenetic modifications leading to *PTEN* inactivation, immunohistochemistry is also very useful for screening for areas of focal PTEN loss. By necessity, *PTEN* FISH is analyzed at high magnification, examining 50-100 nuclei, which may miss small areas of loss within the sampled tumor. In contrast, immunohistochemistry can be easily screened at low magnification and still afford a nearly cell-by-cell resolution image of PTEN expression. In the current study, in over 40% of cases where PTEN immunohistochemistry detected loss and *PTEN* FISH was initially read as 2 copies in the identical tumor core, rescreening the FISH guided by areas of immunohistochemistry loss resulted in detection of small areas with *PTEN* deletion, initially missed or beneath the cutoff for the FISH scoring. This result, in addition to the high negative predictive value of intact immunohistochemistry for lack of deletion strongly suggests that immunohistochemistry screening for PTEN loss is likely to be an efficient and cost-effective strategy to ascertain PTEN status in tissue sections.

Akin to HER2 assessment in breast, it is ultimately likely that the best protocol will be to perform reflexive FISH on a subset of prostate tumors after initial immunohistochemistry screening. Clearly, in cases with ambiguous immunohistochemistry results (<5%), FISH will have an important role. However, there may also be a role for FISH in cases with heterogeneous loss of PTEN by immunohistochemistry. As in previous cohorts (12), in the current cohort we found that homogeneous PTEN immunohistochemistry loss was more strongly associated with decreased recurrence-free survival compared to heterogeneous PTEN protein loss in both univariate and multivariate analyses (26). The explanation for why focal PTEN loss is a less potent prognostic indicator than homogeneous loss remains unclear. Homogeneous PTEN loss may be a surrogate indicator for expansion of a single, dominant clone of tumor cells. Alternatively, perhaps loss of PTEN in a larger number of cells increases risk of tumor progression for stochastic reasons. Finally, this result may also be related to the higher prevalence of homozygous *PTEN* deletion among the cases with homogeneous immunohistochemistry loss, compared to the cases with heterogeneous immunohistochemistry loss (64% vs 26%; $p<0.0001$ by Fisher's exact test). Indeed, in the subset of the current cohort where *PTEN* FISH was correlated with disease outcomes, only

homozygous but not hemizygous *PTEN* loss was associated with decreased recurrence free survival in multivariate models (17). Thus, it may be that tumors with heterogeneous PTEN protein loss and underlying homozygous *PTEN* gene deletion have outcomes roughly equivalent to cases with homogeneous PTEN protein loss (the majority of which have homozygous deletion). Though larger case numbers than were included in the current study will be required to formally address this hypothesis, this would suggest that it may useful to perform reflexive FISH in the case of heterogeneous PTEN protein loss by immunohistochemistry (14% of total cases in current cohort) to determine whether there is underlying homozygous *PTEN* gene deletion. The FISH could be guided by the immunohistochemistry to focus on areas with protein loss, increasing the sensitivity of the assay in this way.

There are a number of limitations of the current study. Though both FISH and immunohistochemistry were performed on the same tissue microarrays, analysis of all tissue microarray cores was not technically feasible for both methods in all cases and correlation between the two assays was done on a tumor-by-tumor rather than core-by-core basis for most cases. Thus, some of the disagreements between FISH and immunohistochemistry likely came about because of tumor heterogeneity, where different areas of the same tumor were being analyzed by each assay, and standard section analysis of a subset of cases largely bears this out. In addition, the gold-standard for assessing *PTEN* gene status is not clear at this point. Though FISH can detect larger deletions which are the most common mechanism of loss in prostate cancer, it will miss smaller deletions, as well as indels and missense mutations which may inactivate the gene. Thus, in cases where the same tumor tissue was analyzed, it is impossible to know the true cause of the apparent discrepancies between FISH and immunohistochemistry without using a third methodology such as sequencing to examine for gene alterations that would be missed by FISH (these studies are ongoing in separate cohorts currently). Finally, due to the relatively small numbers of discordant cases overall, it was not feasible to do a meaningful analysis comparing FISH and immunohistochemistry for prediction of prognosis in these cases, to determine which assay is a better prognostic tool.

In conclusion, in a large multi-institutional cohort of prostate tumors, our immunohistochemistry assay for PTEN loss shows the highest specificity and sensitivity for *PTEN* gene deletion reported for an immunohistochemistry assay to date. These data strongly suggest that immunohistochemistry is a cost-efficient method to screen for PTEN loss in prostate tumors, requiring ~$100 and a single 4 μm tumor section for assay performance. In cases with ambiguous PTEN immunohistochemistry results or heterogeneous PTEN protein loss, reflexive *PTEN* FISH may be a useful confirmatory test. This inexpensive, automated and analytically validated immunohistochemistry assay has already been used to demonstrate the association of PTEN loss with lethal prostate cancer in a large population-based cohort in multivariable models (18). Ultimately, its portability will enable the performance of clinical validation studies on a large number of additional cohorts, credentialing PTEN as a prognostic and potentially predictive biomarker in diverse clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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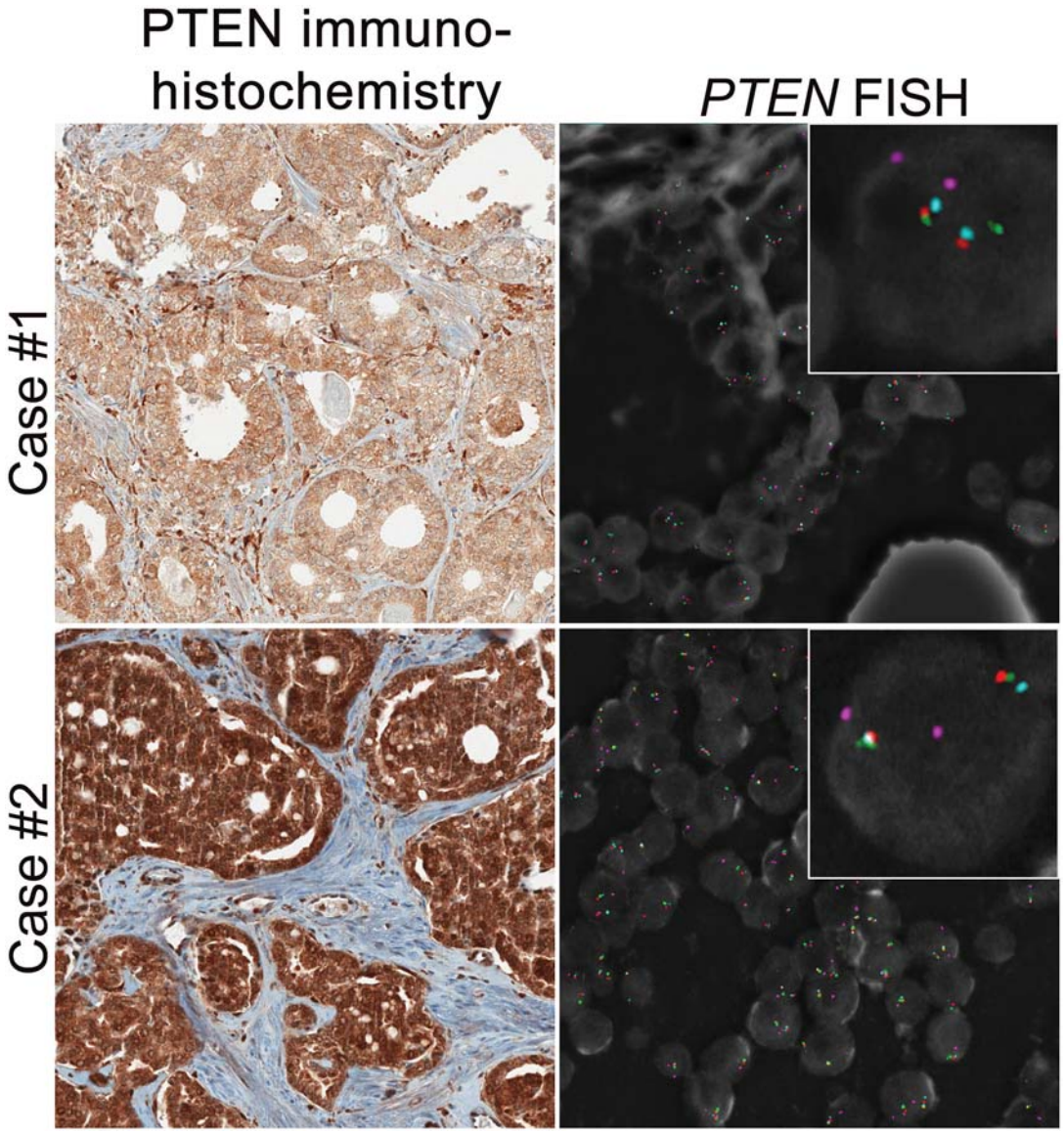

Figure 1.

Prostate cancer cases showing intact PTEN protein with 2 intact PTEN gene alleles. Cases #1 and 2: PTEN immunohistochemistry demonstrates intact PTEN protein (left), while four-color FISH image from adjacent section (right) shows two intact *PTEN* alleles (see enlarged inset—two red signals) with two intact copies flanking genes, *WAPAL* (green) and *FAS* (aqua) as well as chromosome 10 centromeres (pink).

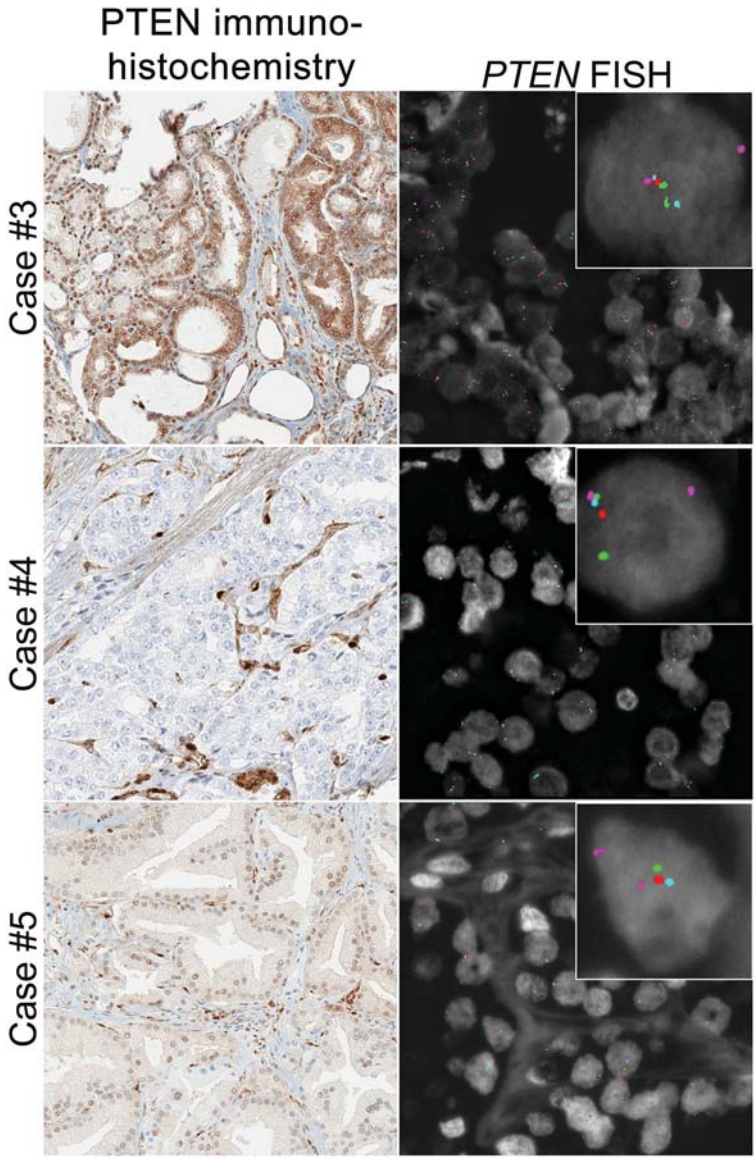

Figure 2.

Prostate cancer cases showing variable PTEN protein expression with hemizygous *PTEN* gene deletion. Case #3: PTEN immunohistochemistry demonstrates intact PTEN protein (left), with four-color FISH image from an adjacent section showing a hemizygous *PTEN* deletion with loss of one *PTEN* gene (see enlarged inset-one red signal). Since both centromeres (pink) and the *WAPAL* (green) and *FAS* (aqua) probes that flank either side of *PTEN* are retained it is likely that this hemizygous deletion is interstitial and restricted to the *PTEN* region. Case #4: PTEN immunohistochemistry image shows homogeneous loss of PTEN protein (left) while FISH image from an adjacent section (right) shows a hemizygous *PTEN* deletion (see enlarged inset-one red signal). Concurrent hemizygous deletion of the adjacent *FAS* gene probe (one aqua signal missing) but retention of two copies of the centromere and *WAPAL* gene probes indicates the deletion includes both the *PTEN* and *FAS* genes. Case #5: PTEN immunohistochemistry image shows somewhat light, but intact

immunostaining for PTEN protein (left) while the FISH image from an adjacent section (right) shows a hemizygous *PTEN* deletion (see enlarged inset-one red signal). Since there was concurrent loss of the *WAPAL, PTEN* and *FAS* gene probes (green, red and aqua, respectively), but retention of both centromeres (pink), this hemizygous deletion extends outside the *PTEN* region in both directions.

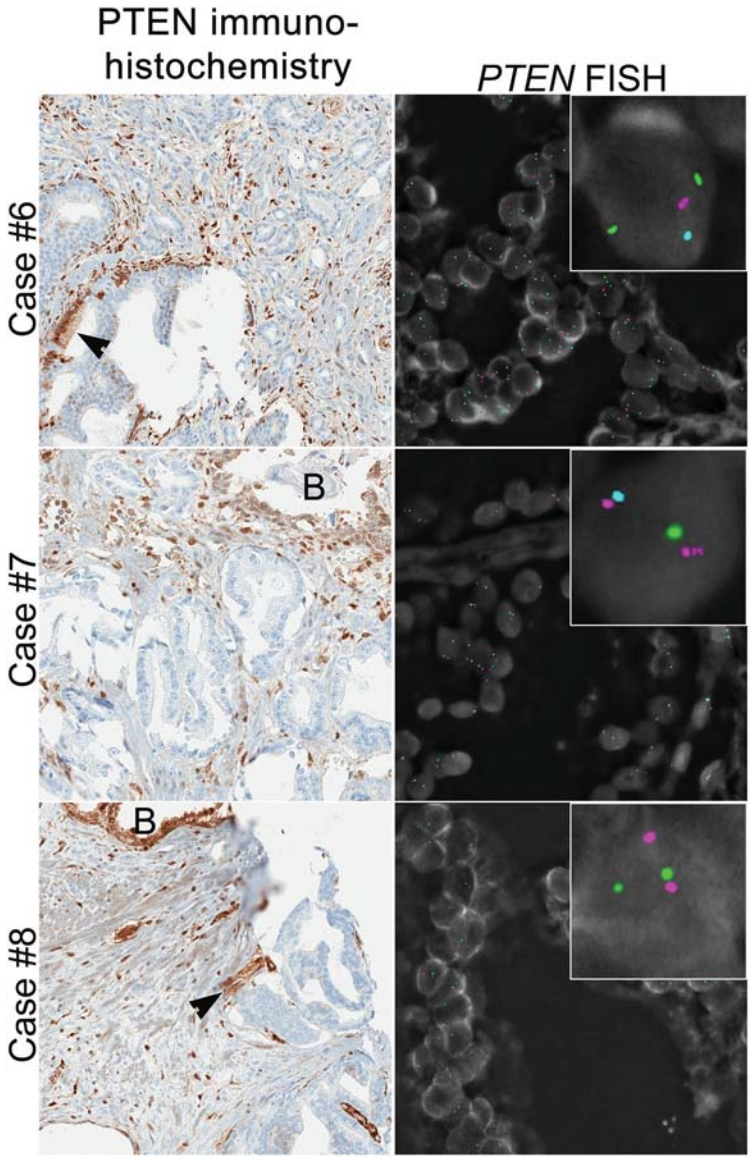

Figure 3.

Prostate cancer cases showing absence of PTEN protein expression with homozygous *PTEN* gene deletion. Case #6: PTEN immunohistochemistry image (left) shows loss of PTEN protein in tumor glands. Intraductal spread of tumor is present in this case and retention of PTEN protein is seen in benign basal and luminal cells of duct containing tumor (arrowhead). Four-color FISH image from an adjacent section (right) shows a homozygous deletion with loss of both *PTEN* genes (see enlarged inset - no red signals). The retention of the centromeres (pink) and both *WAPAL* genes (green), but the presence of only one copy of the *FAS* gene (aqua) indicates that one of the deletions involved both the *PTEN* and *FAS* genes. Case #7: PTEN immunohistochemistry image (left) shows loss of PTEN protein in tumor glands, with retention in entrapped benign gland (B). FISH image from an adjacent section (right) shows a homozygous *PTEN* deletion (see enlarged inset - no red signals). The retention of the centromeres (pink) but concurrent loss of one *WAPAL* (green) and one *FAS*

gene (blue) indicates the deletions extend outside the *PTEN* region. Case #8: PTEN immunohistochemistry image (left) shows loss of PTEN protein in tumor glands, with retention in adjacent benign gland (B) and nearby endothelial cells (arrowhead). (FISH image from an adjacent section (right) shows a homozygous *PTEN* deletion (see enlarged inset - no red signals). The retention of the centromeres and both the *WAPAL* genes (green), but the concurrent loss of both *FAS* (blue) and *PTEN* (red) indicates that both copies of chromosomes 10 have deletions involving these genes.

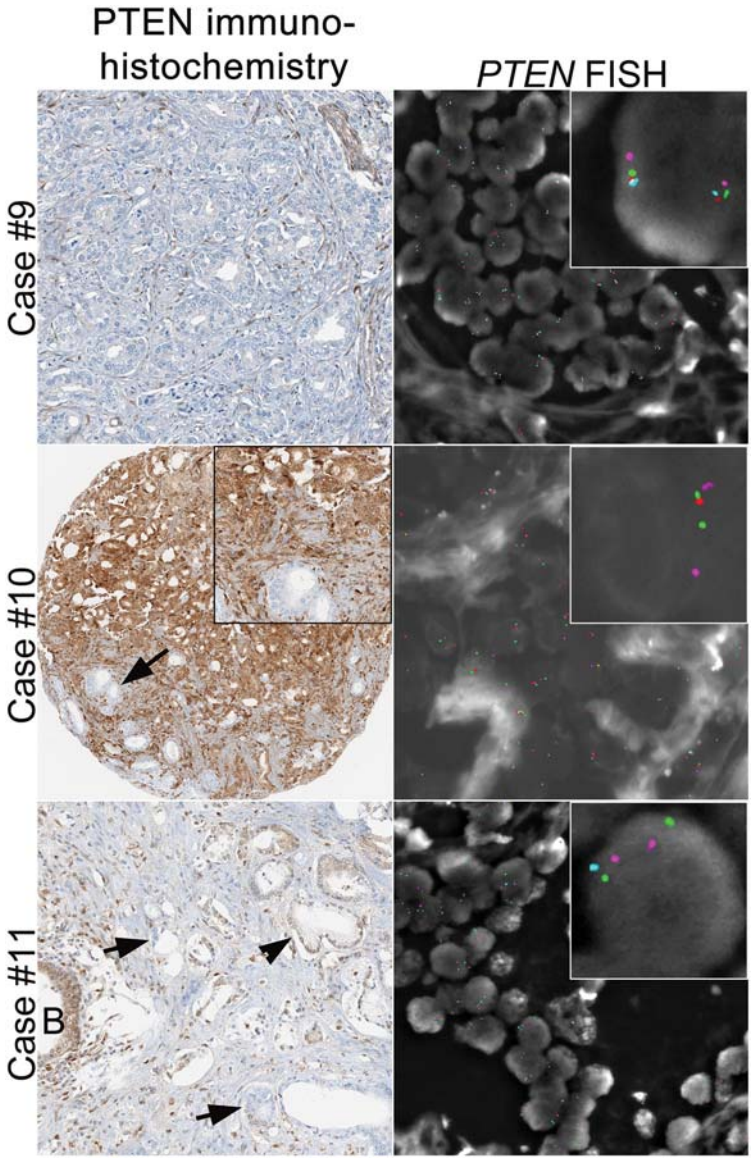

Figure 4A.

Prostate cancer cases with discordant PTEN immunohistochemistry and FISH results on initial review. Case #9: PTEN immunohistochemistry demonstrates very weak cytoplasmic immunostaining with loss of nuclear immunostaining and thus was called negative on initial review, though in retrospect it may be better classified as ambiguous due to weak staining and absence of benign glands for comparison (left). Four-color FISH image from an adjacent section that is representative of all examined cores in this tissue microarray (right) indicates that the *PTEN* gene does not have a detectable deletion by FISH. The enlarged inset shows that the centromeres, *WAPAL, PTEN* and *FAS* gene probes are each present as two copies. Case #10: PTEN immunohistochemistry image (left) shows heterogeneous PTEN loss in some tumor glands (arrow) but PTEN protein is expressed by majority of other tumor glands in this core. FISH image from an adjacent section (right) was initially read as PTEN intact, but shows a focal area with hemizygous *PTEN* deletion recognized on re-examination

guided by immunohistochemistry. The enlarged inset shows there is only one copy of the red *PTEN* gene probe (one red signal) and loss of both aqua *FAS* gene probes. Case #11: PTEN immunohistochemistry image (left) demonstrates heterogeneous PTEN loss in some tumors glands (arrows) but not in others (arrowheads). FISH image from an adjacent section (right) shows the small area of the section that had a homozygous *PTEN* deletion on re-examination. The enlarged inset shows that there are no copies of the red *PTEN* gene probe and one copy of the aqua *FAS* gene probe, but retention of the adjacent *WAPAL* and centromere probes.

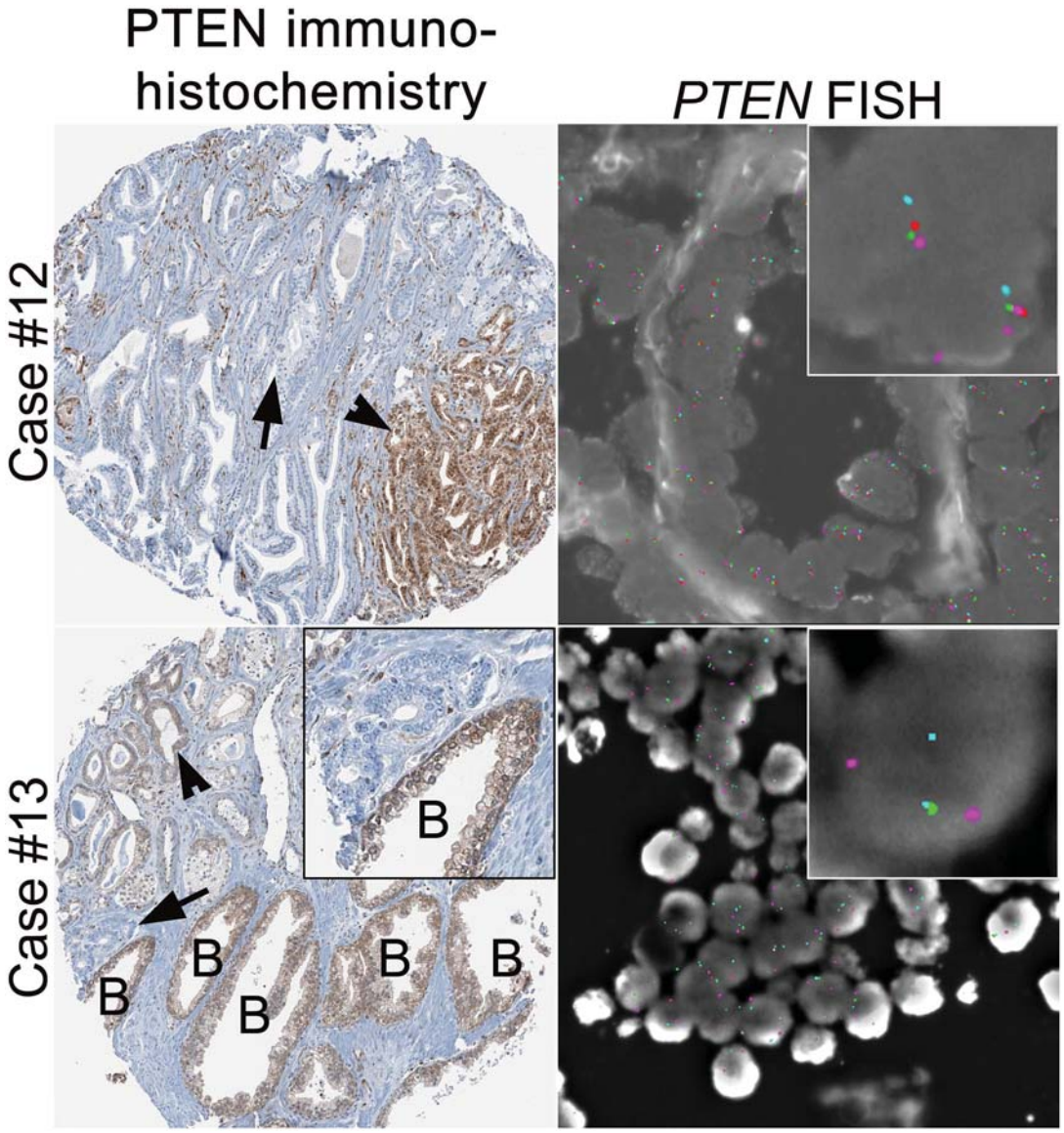

Figure 4B.

Prostate cancer cases with discordant PTEN immunohistochemistry and FISH results on initial review. Case #12: PTEN immunohistochemistry image (left) shows heterogeneous loss of PTEN protein in some tumor glands (arrow) but not in others (arrowhead). A FISH image from an adjacent section that is representative of all examined cores in this tissue microarray (right) indicates that the *PTEN* gene does not have a detectable deletion by FISH. The enlarged inset shows that the centromeres, *WAPAL, PTEN* and *FAS* gene probes are each present as two copies. The heterogeneous loss in this case may have resulted in different tumor areas sampled in slides for immunohistochemistry and that for FISH. Case #13: PTEN immunohistochemistry image (left) shows predominantly intact/light immunostaining in tumor glands (arrowhead) and benign glands (B) with a very focal area of tumor with PTEN loss identified on re-review after FISH analysis (arrowhead, inset). FISH analysis of an adjacent section to the immunohistochemistry indicates a homozygous

PTEN deletion. The enlarged inset shows that there are no copies of the red *PTEN* gene probe and loss of one green *WAPAL* gene probe but retention of both the *FAS* and the centromere probes.

Table 1

Summary of PTEN immunohistochemistry by PTEN FISH status.

	PTEN FISH					
	Intact		Hemi-deletion		Homo-deletion	
	N	%	N	%	N	%
PTEN immunohistochemistry						
Intact	549	91	22	35	2	3
Heterogeneous loss	45	7	26	42	25	37
Homogeneous loss	8	1	14	23	40	60

Table 2

Performance metrics for PTEN immunohistochemistry compared to gold-standard *PTEN* FISH

	%	n
Specificity	91	549/602
Sensitivity for homozygous deletion	97	65/57
Sensitivity for hemizygous deletion	65	40/62
Positive predictive value	66	105/158
Negative predictive value	96	549/573

Table 3

Comparison of PTEN immunohistochemistry and FISH results on tissue microarray cores and standard tissue section slides

Case	Tissue microarray PTEN immunohistochemistry	standard slide PTEN immunohistochemistry	Tissue microarray *PTEN* FISH	standard slide *PTEN* FISH
1	intact	intact	intact	intact
2	intact	heterogeneous loss	intact	intact
3	intact	intact	intact	intact
4	intact	intact	intact	failure
5	heterogeneous loss	heterogeneous loss	homo-deletion	hemi-deletion and homo-delletion
6	heterogeneous loss	heterogeneous loss	homo-deletion	homo-deletion
7	heterogeneous loss	heterogeneous loss	homo-deletion	homo-deletion
8	heterogeneous loss	heterogeneous loss	intact	intact
9	heterogeneous loss	heterogeneous loss	intact	intact
10	heterogeneous loss	intact	intact	intact
11	heterogeneous loss	heterogeneous loss	intact	intact
12	heterogeneous loss	intact	intact	hemi-deletion
13	heterogeneous loss	heterogeneous loss	intact	homo
14	heterogeneous loss	heterogeneous loss	intact	homo
15	heterogeneous loss	heterogeneous loss	intact	hemi-deletion of *WAPAL*
16	homogeneous loss	homogeneous loss	hemi-deletion	hemi-deletion
17	homogeneous loss	heterogeneous loss	intact	hemi-deletion
18	ambiguous	intact	intact	intact
19	heterogeneous loss	heterogeneous loss	core missing	intact
20	heterogeneous loss	heterogeneous loss	core missing	intact

Loss of Expression of AZGP1 Is Associated With Worse Clinical Outcomes in a Multi-Institutional Radical Prostatectomy Cohort

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BACKGROUND. Given the uncertainties inherent in clinical measures of prostate cancer aggressiveness, clinically validated tissue biomarkers are needed. We tested whether Alpha-2-Glycoprotein 1, Zinc-Binding (AZGP1) protein levels, measured by immunohistochemistry, and RNA expression, by RNA in situ hybridization (RISH), predict recurrence after radical prostatectomy independent of clinical and pathological parameters.
METHODS. AZGP1 IHC and RISH were performed on a large multi-institutional tissue microarray resource including 1,275 men with 5 year median follow-up. The relationship between IHC and RISH expression levels was assessed using the Kappa analysis. Associations with clinical and pathological parameters were tested by the Chi-square test and

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Conflicts of interest: None.

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the Wilcoxon rank sum test. Relationships with outcome were assessed with univariable and multivariable Cox proportional hazards models and the Log-rank test.
RESULTS. Absent or weak expression of AZGP1 protein was associated with worse recurrence free survival (RFS), disease specific survival, and overall survival after radical prostatectomy in univariable analysis. AZGP1 protein expression, along with pre-operative serum PSA levels, surgical margin status, seminal vesicle invasion, extracapsular extension, and Gleason score predicted RFS on multivariable analysis. Similarly, absent or low AZGP1 RNA expression by RISH predicted worse RFS after prostatectomy in univariable and multivariable analysis.
CONCLUSIONS. In our large, rigorously designed validation cohort, loss of AZGP1 expression predicts RFS after radical prostatectomy independent of clinical and pathological variables. *Prostate 76:1409–1419, 2016.*

KEY WORDS: **prostate cancer; AZGP1; immunohistochemistry; prognosis**

INTRODUCTION

Despite decreasing prostate cancer death rates over the past decade, systematic screening with serum prostate specific antigen (PSA) testing has been heavily criticized [1,2]. Two large randomized trials (PLCO and ERSPC) have noted little or no survival benefit derived from PSA testing and suggested that prostate cancer is over treated [3,4]. Paralleling these trials has been a growing realization that low risk prostate cancers left untreated can often show an indolent clinical course, giving rise to the concept of active surveillance for low risk lesions [5]. Long-term follow-up from several active surveillance cohorts suggests that this is a safe approach, although not entirely without risk [6–8]. Virtually, all of the active surveillance programs involve relatively intense testing with PSA, digital rectal examinations, repeat biopsies, and more recently, MRI examinations [9–11]. This follow-up is necessitated by the inability to characterize the biological potential of low risk prostate cancers, as well as by sampling errors in biopsy and the poor performance of clinical measures of tumor aggressiveness. This follow-up also incurs significant financial and human costs due to repeated testing. Complicating matters further, some localized prostate cancers treated with surgery or radiation therapy alone appear to be more aggressive than clinical and pathological features suggest, and these might benefit from adjuvant therapy. Given the risks of over-treatment and under treatment in localized prostate cancer, new biomarkers to help characterize tumor aggressiveness are needed.

To address this need, our group has assembled a retrospective cohort of patients who have undergone radical prostatectomies and who have long-term follow-up [12]. Using a case-control design with a quota-sampling plan, we have constructed a multi-institutional tissue microarray (TMA) resource for validation of candidate biomarkers of clinical outcome, with both pathological and clinical outcomes, such as recurrence free survival, recorded for nearly all patients [13,14].

We have previously demonstrated that loss of expression of zinc-alpha 2-glycoprotein (AZGP1 or ZAG) protein expression by immunohistochemistry is associated with an increased risk of recurrence after radical prostatectomy [15]. This finding has been validated in several later studies, and loss of AZGP1 expression has also been shown to predict subsequent development of metastatic disease and death from prostate cancer [16–19]. In addition, loss of transcriptional expression of AZGP1 has been associated with prostate cancer recurrence and death and is 1 of 12 prognostic transcripts measured in a commercially available tissue-based test called OncotypeDX from Genomic Health [20]. OncotypeDX scores have been shown to correlate with adverse pathology on low and intermediate risk patients undergoing radical prostatectomy [20].

Our objective is to validate candidate biomarkers of prognosis to aid in treatment selection for men with localized prostate cancer. Based on strong preliminary data implicating loss of AZGP1 expression as a marker of adverse outcome in prostate cancer, we tested whether loss of RNA expression, using chromogenic RNA in situ hybridization (RISH), and loss of protein expression, by immunohistochemistry, were associated with recurrence free survival after radical prostatectomy.

MATERIALS AND METHODS

TMA Cases and Construction

The study was carried out under IRB-approved protocols at each participating site (Stanford University, University of California San Francisco, University of Washington, University of British Colombia, University of Texas Health Sciences Center at San

Antonio, Eastern Virginia Medical Center) and a materials transfer agreement that allowed sharing of tissue microarrays, clinical information and tissue samples. Cases included in the TMA cohort were selected randomly by the study statistician (ZF) using de-identified clinical data from each site such that recurrent and non-recurrent cases were balanced. Constraints were placed on selection such that recurrent cases in patients with Gleason score $3+3=6$ and non-recurrent cases in those with Gleason score $4+4=8$ were oversampled. Details of case selection, tissue microarray construction, and statistical considerations have been detailed elsewhere [12].

TMAs were constructed at six participating centers using agreed upon standard operating procedures and TMA layouts [12]. Briefly, 3 cores of the highest grade cancer from the largest cancer area were harvested as 1 mm cores and transferred to the recipient block. In addition, 1 core of histologically normal prostate tissue was included from each case. A common set of tissue cores (colon, tonsil, kidney, healthy prostate, liver) from a single study site were placed in each TMA block as a staining control and for normalization. Once constructed, the TMAs were baked and stored under nitrogen gas at each site.

Immunohistochemistry

Freshly cut 5 μ sections from each site were shipped to Stanford University for immunohistochemical staining. AZGP1 immunohistochemistry was performed using a commercial antibody (1:1500 dilution; HPA012582, Sigma–Aldrich). All stained slides were digitalized using the Leica SCN400 scanning system with the SL801 autoloader (Leica Microsystems; Concord, Ontario, Canada) at magnification equivalent to 40×. The images were exported and stored in the SlidePath digital imaging hub (DIH; Leica Microsystems). Separate TMA sections were stained with hematoxylin and eosin (H & E) and high molecular weight keratins (HMWK, 34bE12, Dako) and scored for the presence of cancer in each core on the TMA as described previously [13]. AZGP1 protein and RNA staining were scored by a single pathologist (LF) only in cores in which cancer was present as determined using the H & E and HMWK stains.

Representative cores (clearly positive, clearly negative, and mixed positive/negative) were manually identified and values on a four-point scale were assigned to each immunostain and RISH. Immunohistochemical staining for AZGP1 was defined as absent, weak (faint cytoplasmic staining of scattered cells), moderate (intermediate or heterogeneous cytoplasmic staining in tumor cells), and strong (dense cytoplasmic staining of nearly all tumor cells) as defined previously [15]. Similarly, AZGP1 RISH staining was scored as absent, weak, moderate, and strong.

RNA In Situ Hybridization (RISH)

AZGP1 RNA expression was performed on 5 μ sections using the RNAscope® 2.0 HD Detection Kit (Red) assay (Cat. No. 310034, Advanced Cell Diagnostics) using probes for AZGP1 (Advanced Cell Diagnostics). Sections were deparaffinized in a series of xylene and ethanol and allowed to dry before incubation with "pretreatment 1" for 10 min at room temperature, boiled in "pretreatment 2" for 15 min, and protease-digested in "pretreatment 3" at 40°C for 30 min. Slides were then processed according to the manufacturer's instructions. The bacterial gene DapB was used as a negative control and the housekeeping gene POLR2A served as a positive control. Sections were counterstained with Gill's hematoxylin (Sigma–Aldrich) and mounted with Ecomount (Biocare Medical).

Statistical Methods

A total of 1,326 subjects are represented on the TMA and had their clinical data collected. The clinical and pathological characteristics included in the analysis were age, pre-surgery PSA, post-surgical Gleason score, seminal vesicle invasion (SVI), extra-capsular invasion (ECE), and surgical margin status. Subjects with 25% or more of their clinical or pathological characteristics missing were excluded from this analysis ($N=51$). A total of 1,275 patients with evaluable AZGP1 staining data and the clinical and pathological data were included in the analysis.

The primary endpoint of this analysis was post-surgical recurrence-free survival (RFS) defined as the absence of PSA (biochemical) recurrence, local recurrence, prostate cancer metastases, or death from prostate cancer, with events scored at the earliest date noted after surgery. Disease-specific survival (DSS), defined as death from prostate cancer or development of advanced metastatic disease, and overall survival (OS) were secondary endpoints. For all endpoints the baseline was set at the date of surgery. AZGP1 IHC and RISH score for each patient was the maximum score of all the cores from that patient as defined above. Based on previous work, AZGP1 stained cases were grouped as negative/weak staining and compared to moderate/strong staining [15].

Summary statistics of patients' AZGP1 protein and RNA scores were provided in frequencies and percentages. The association between AZGP1 expression levels by IHC and RISH was assessed by Kappa analysis and the Chi-square test. The association between AZGP1 expression levels and categorical

values (seminal vesicle invasion, extracapsular extension, and positive surgical margins) was assessed by Chi-square test. The association between AZGP1 expression and continuous variables (pre-operative serum PSA levels and age) was assessed by the Wilcoxon rank sum test. The Kaplan–Meier (KM) method was used to estimate survival endpoints by AZGP1 expression group. Cox proportional hazard model was used to estimate effects AZGP1 expression on each survival endpoint. Unweighted and weighted analyses were performed, with the latter accounting for the oversampling of patients with recurrence less than 5 years after surgery. All tests were two-sided and *P*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using SAS version 9 (SAS Institute, Cary, NC). Kaplan–Meier plots were generated using Spotfire S+ 8.2 (TIBCO Inc., Palo Alto, CA).

RESULTS

Patient Population and Staining Results

A total of 1,275 patients had available clinical and pathological data, as well as evaluable AZGP1 expression status by either IHC or RISH (representative images shown in Fig. 1). For AZGP1 IHC, a total of 139 cases (11%) did not have evaluable staining data either because of core loss or because lack of cancer in the core samples. Of the remaining tumors, 22% (252/1,136) showed absent expression, 21% (240/1,136) showed weak expression, 33% (372/1,136) showed moderate expression, and 24% (272/1,136) showed strong expression. The distribution of AZGP1 RISH staining was very similar—absent expression: 23%; weak: 32%; moderate: 24%; and strong: 22%. For RISH, 186 cases did not have evaluable AZGP1 staining. AZGP1 expression levels, clinical, and pathological data are summarized in Table I.

TABLE I. Summary of Pathological Characteristics

Variable	Status	Number	Percentage
Gleason score	Missing	10	0.78
	≤6	549	43.06
	3 + 4 = 7	458	35.92
	4 + 3 = 7	143	11.22
	8–10	115	9.02
Extracapsular extension	Missing	17	1.33
	No	877	68.78
	Yes	381	29.88
Seminal vesicle invasion	Missing	17	1.33
	No	1,177	92.31
	Yes	81	6.35
Surgical margins	Missing	179	14.04
	Positive	385	30.20
	Negative	711	55.76
AZGP1 protein IHC	Missing	139	10.90
	Absent	252	19.76
	Weak	240	18.82
	Moderate	372	29.18
	Strong	272	21.33
AZGP1 RISH	Missing	186	14.59
	Absent	252	19.76
	Weak	344	26.98
	Moderate	257	20.16
	Strong	236	18.51

Expression levels of AZGP1 protein measured by IHC and RNA by RISH were associated with each other, although the correlation was modest. When compared on a per core basis, the Kappa value for the four staining groups between IHC and RISH was only 0.15 (95%CI: 0.12–0.18), although the correlation by Chi-square test was highly significant ($P < 0.0001$). When IHC and RISH were assessed for each patient by grouping results on the 3 cores for each patient tumor sample the Kappa improved to 0.34 (95%CI:

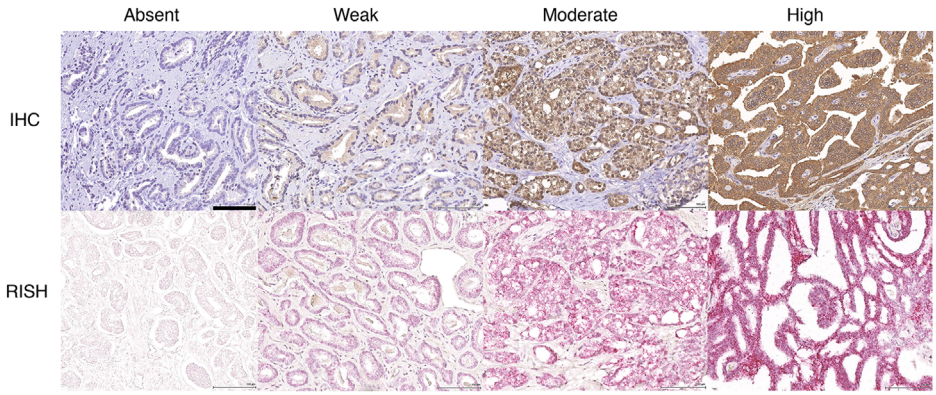

Fig. 1. Immunohistochemical and RISH AZGP1 staining in representative prostate cancer samples showing absent, weak, moderate, and strong staining. Black bar in the upper left micrograph corresponds to 100 μ. All images scaled the same.

0.30–0.38) and the Chi-square test remained highly significant ($P < 0.0001$). Grouping patient samples into absent/weak compared to moderate/strong expression did not improve the correlation between RISH and IHC (Kappa: 0.34; 95%CI: 0.29–0.40; $P < 0.0001$ by Chi-square test).

AZGP1 Expression and RFS After Radical Prostatectomy

Previous reports have demonstrated that AZGP1 expression assessed by IHC is prognostic in prostate cancer when cases are split categorically into absent/weak expression compared to moderate/high level expression [15,17]. Our objective was to test whether splitting samples in this fashion could be validated in our carefully selected cases of patients who had undergone radial prostatectomy and had associated detailed clinical data including long-term follow-up. Kaplan–Meier analysis demonstrated that absent and weak expression of AZGP1 protein showed significantly worse RFS compared to moderate and high expressing tumors (Supplementary Fig. S1A). Moreover, RFS for the absent and weak staining were virtually identical as was RFS for the moderate and high level expression categories. These findings validated previous groupings of staining into two categories for IHC and therefore samples were divided into absent weak versus moderate/strong for the remaining analyses.

AZGP1 expression levels measured by RISH showed a similar pattern to those seen with IHC, although with some differences (Supplementary Fig. S1B). RISH expression level was a weaker predictor of RFS after prostatectomy ($P = 0.011$, log-rank test) compared to IHC ($P < 0.0001$, log-rank test). In addition, AZGP1 expression level assessed by RISH did not segregate into two discrete groups. While RFS appeared to be similar between absent and weak staining by RISH, moderate staining appeared to have intermediate outcomes compared to these groups and those that expressed high levels of AZGP1 RNA. However, the differences in RFS between moderate expressing cases and high and low expressing cases was small. Therefore, to allow for comparison of IHC and RISH results, we grouped RISH cases into absent/weak and moderate/strong staining.

AZGP1 Protein and RNA Expression and Clinicopathological Features

AZGP1 levels by IHC and RISH were tested for their association with clinical and pathologic features (Table II). For both IHC and RISH, absent/weak expression of AZGP1 was associated with adverse clinical features including positive surgical margins (PSM), extracapsular extension (ECE), and higher Gleason score (GS). However, absent/weak expression was not associated with seminal vesicle invasion (SVI), pre-operative serum PSA levels, or patient age (all $P > 0.05$). Lymph node status was not available for approximately half of the cases, and therefore, was not included in the analysis. Taken together, AZGP1 expression status is associated with many, but not all clinical features important in prostate cancer prognosis after surgery.

AZGP1 Expression and Clinical Outcomes

In univariable Cox proportional hazards analysis, absent or weak staining for AZGP1 by IHC was associated with significantly worse RFS (HR = 1.49; 95%CI 1.26, 1.77; $P < 0.0001$). Absent/weak AZGP1 expression was also associated with worse DSS (HR = 1.84; $P = 0.03$) and OS (HR = 1.94; $P = 0.01$). Likewise, absent/weak expression of AZGP1 by RISH

TABLE II. AZGP1 Staining Status and Pathological Parameters

Feature	Status	IHC low	IHC high	*P*-value	RISH low	RISH high	*P*-value
Surgical margins	Yes	166	181	0.004	199	131	0.006
	No	242	389		306	297	
SV invasion	Yes	38	35	0.14	43	27	0.27
	No	448	599		547	457	
Extracaps exten	Yes	169	173	0.004	213	116	<0.0001
	No	313	467		375	372	
Gleason score	≤6	189	281	0.003	272	181	0.009
	3 + 4 = 7	177	243		209	192	
	4 + 3 = 7	54	74		56	70	
	8–10	66	45		56	46	

P-values by Chi-square test.

was associated with worse RFS, although to a lesser degree (HR = 1.26; 95%CI 1.05, 1.50; $P = 0.01$). However, AZGP1 expression by RISH was not associated with DSS or OS ($P = 0.32$ and $P = 0.26$, respectively). Univariable Cox proportional hazards analysis for AZGP1 expression levels and clinical and pathological data are summarized in Table III. Kaplan–Meier analysis demonstrated that absent/weak expression AZGP1 IHC expression compared to moderate/high expression was significantly associated with RFS ($P < 0.0001$, log-rank test), overall survival ($P = 0.013$, log-rank test), and disease specific survival ($P = 0.024$, log-rank test) (Fig. 2A–C). Absent/weak expression AZGP1 RNA by RISH was also associated with worse RFS ($P = 0.011$, log-rank test), but not with OS or DSS (Fig. 2D–F).

To evaluate whether AZGP1 IHC or RISH expression levels provided prognostic information independent of clinical variables, we performed multivariable Cox proportional hazards analysis using a backwards elimination procedure to identify the final model for each endpoint (Table IV). For RFS, absent/weak AZGP1 expression levels assessed by either IHC or RISH were independently associated with worse clinical outcome (HR = 1.39; $P = 0.002$ and HR 1.28; $P = 0.02$, respectively), as were presence of positive surgical margins, extracapsular extension, seminal vesicle invasion, higher pre-operative PSA, and increasing Gleason score. The concordance index (C-index) for the model including margins, SVI, GS, and log(PSA) was 0.656 and improved to 0.659 with the addition of ECE, or to 0.661 with the addition AZGP1 RISH. A model including margins, SVI, ECE, GS, log(PSA) improved the C-index from 0.659 to 0.665 with addition of AZGP1 IHC. However, AZGP1 expression assessed either by IHC or RISH was not associated with DSS or OS on multivariable analysis. DSS was associated only with Gleason score and pre-operative PSA and OS survival was associated only with Gleason score and age as we have reported previously [13]. The relatively small number of prostate cancer deaths or metastases (n = 54) or deaths from all causes (n = 71) limited our ability to test the association of the biomarkers with these endpoints.

Given our interest in identifying prognostic biomarkers for selection of patients for active surveillance, we evaluated whether AZGP1 expression could predict outcome in patients with GS $\leq 3+3=6$. In univariable Cox proportional hazards analysis, AZGP1 IHC (HR 1.8) and RISH (HR 1.9) remained significant predictors of outcome. In a multivariable model including PSA, SVI, ECE, and SM, both remained significant (HR 1.7 for both) (Supplemental Table SI). Kaplan–Meier analysis confirmed strong association of AZGP1 RISH and IHC with RFS (Supplemental Fig. S2). The C-index improved from 0.618 to 0.662 for IHC and 0.658 for RISH over the clinical model that included PSA, SVI, ECE, and SM for patients with GS $\leq 3+3=6$.

DISCUSSION

There are no immunohistochemical markers of prognosis in clinical use to aid in the management of prostate cancer, despite clear clinical needs, and despite the number of candidates reported in the literature. One recurring issue has been the lack of meaningful validation for many biomarkers [21]. To address this need, we developed the Canary multi-institutional TMA with the explicit design to validate candidate biomarkers of prognosis in clinically localized low and intermediate risk disease [12]. Using this platform, we have validated PTEN copy alterations using FISH and PTEN protein expression using a clinical grade assay, and Ki67 staining as providing prognostic information independent of clinical and

TABLE III. Univariable Cox Proportional Hazards Model for Recurrence Free Survival

Factor	Comparison	Hazard ratio	95% LCL	95% UCL	*P*-value	#Event	#Censored	Total #pts
AZGP1 IHC	Negative/weak versus moderate/strong	1.49	1.26	1.77	<0.0001	521	615	1,136
AZGP1 CISH	Negative/weak versus moderate/strong	1.26	1.05	1.50	0.01	506	583	1,089
Margin	Pos versus neg	2.08	1.74	2.48	<0.0001	495	601	1,096
SVI	Pos versus neg	3.38	2.61	4.38	<0.0001	568	690	1,258
ECE	Pos versus neg	1.92	1.62	2.27	<0.0001	571	687	1,258
Gleason	3 + 4 versus ≤6	1.43	1.18	1.74	0.0003	570	695	1,265
	4 + 3 versus ≤6	2.39	1.87	3.06	<0.0001			
	8–10 versus ≤6	2.39	1.82	3.13	<0.0001			
Age	1 unit increase	1.00	0.99	1.01	0.65	559	610	1,169
Log(PSA)	1 unit increase	1.87	1.63	2.15	<0.0001	531	616	1,147

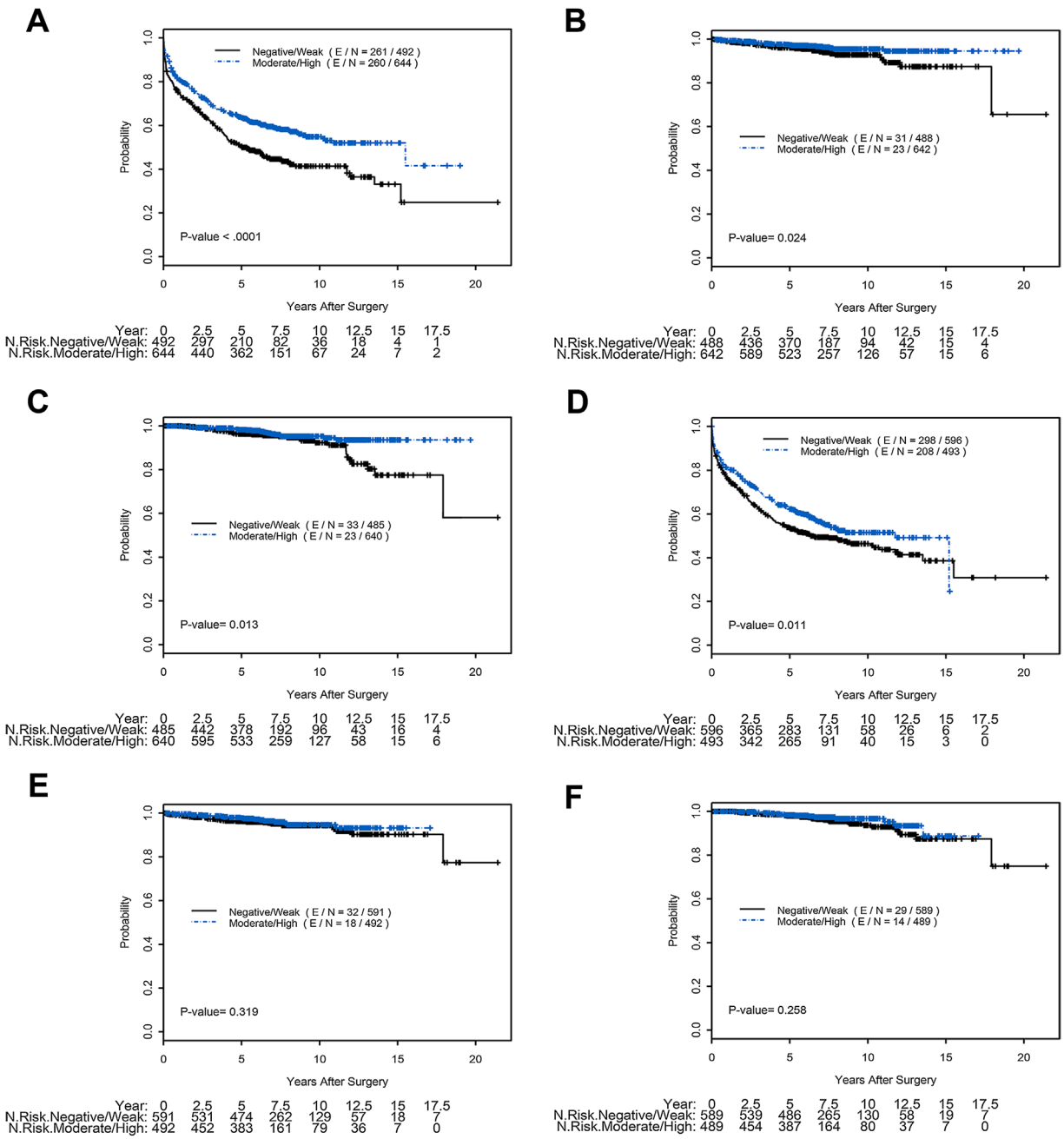

Fig. 2. Kaplan–Meier plots of clinical outcome with staining categorized as absent/weak versus moderate/high AZGP1 expression levels: (**A**) AZGP1 IHC and recurrence free survival; (**B**) AZGP1 IHC and disease specific survival; (**C**) AZGP1 IHC and overall survival; (**D**) AZGP1 RISH and recurrence free survival; (**E**) AZGP1 RISH and disease specific survival; and (**F**) AZGP1 RISH and overall survival.

pathological variables in our tumor set [14,22,23]. Furthermore, we have shown that ERG and SPINK1 protein expression are not predictive of clinical outcome [13]. Here, we demonstrate that AZGP1 protein and RNA expression provide independent prediction of RFS after prostatectomy. Furthermore, AZGP1 protein expression by itself correlates with OS as well as DSS and metastases. Since AZGP1 protein expression shows greater hazard ratios, more significant *P*-values and is readily measured in nearly all pathology laboratories, AZGP1 IHC should be further developed for use in clinical practice, possibly as part of a panel of IHC prognostic biomarkers.

Several previous studies have nominated AZGP1 as a candidate biomarker of prognosis. The role of AZGP1 in prostate cancer prognosis was first identified by our group based on the observation that AZGP1 expression was highly correlated with a

TABLE IV. Multivariable Cox Proportional Hazards Model for Recurrence Free Survival

Factor	Comparison	Hazard ratio	95% LCL	95% UCL	*P*-value
AZGP1 IHC	Negative/weak versus moderate/strong	1.39	1.13	1.71	0.002
Log(PSA)	1 unit increase	1.43	1.21	1.68	<0.0001
Margin	Pos versus neg	1.62	1.31	2.02	<0.0001
SVI	Pos versus neg	2.20	1.58	3.06	<0.0001
ECE	Pos versus neg	1.26	1.01	1.58	0.04
Gleason	3 + 4 versus ≤6	1.19	0.93	1.52	0.16
	4 + 3 versus ≤6	1.99	1.47	2.69	<0.0001
	8–10 versus ≤6	1.43	1.02	1.99	0.04
AZGP1 CISH	Negative/weak versus moderate/strong	1.28	1.04	1.58	0.02
Log(PSA)	1 unit increase	1.46	1.24	1.73	<0.0001
Margin	Pos versus neg	1.71	1.39	2.12	<0.0001
SVI	Pos versus neg	2.26	1.62	3.15	<0.0001
Gleason	3 + 4 versus ≤6	1.22	0.96	1.57	0.11
	4 + 3 versus ≤6	2.12	1.57	2.86	<0.0001
	8–10 versus ≤6	1.60	1.15	2.23	0.006

gene-expression subtype of prostate cancer comprised of low risk tumors with favorable outcome [15]. In addition, we showed that moderate/high AZGP1 protein expression was associated with improved RFS on an independent dataset of prostate tumors that did not overlap with cases included in the current study. Subsequently, several groups have demonstrated that AZGP1 protein expression assessed by IHC is correlated with recurrence-free survival in univariable and multivariable analyses [16,18,19]. Furthermore, Henshall et al. showed that loss of expression of AZGP1 by IHC was associated with the development of metastatic disease in 228 men after prostatectomy, in agreement with our findings [17]. Recently Burdelski et al. have shown that AZGP1 protein expression is a strong independent predictor of clinical outcome in a set of 8,510 patients operated on in Germany [24]. Given the relative strengths of their and our studies, AZGP1 appears to be a highly validated biomarker of prognosis in prostate cancer.

Decreased levels of AZGP1 RNA expression have also been associated with adverse clinical outcome in prostate cancer. AZGP1 transcript levels have been shown in meta-analyses and cross-validation studies to correlate with RFS after radical prostatectomy [15,20,25]. In a recent tiered approach to biomarker identification and validation, AZGP1 was identified as 1 of a set of 12 transcripts that predicts outcome in radical prostatectomy patients, including metastases [20]. Analysis of pre-treatment biopsies using a commercial test using these 12 transcripts and 5 control genes has been shown to predict upgrading and upstaging in men undergoing radical prostatectomy. Ours is the first study to assess AZGP1 RNA expression levels by RISH where we found it remains an independent biomarker of prognosis. While the correlation between RNA and protein levels was modest, they were highly significant, suggesting that AZGP1 expression is regulated largely at the transcript level. However, whereas protein expression appears to show a threshold between weak and moderate staining in influencing outcome, the data from RISH and RNA expression studies do not disclose such a threshold, and RNA expression levels appear to be a continuous predictor of risk. Furthermore, RNA expression measured by RISH was a weaker predictor of outcome compared to AZGP1 IHC. However, RISH is a relatively non-quantitative measure of RNA expression, and it is unclear whether measurement of AZGP1 RNA expression using a more quantitative assay by itself or in the context of other genes will better predict outcome compared to AZGP1 IHC. Direct comparison on identical samples will be necessary to evaluate the relative performance of quantitative measures of AZGP1 RNA and AZGP1 IHC.

Our study, coupled with previous work, strongly suggests that AZGP1 IHC could have value as a prognostic biomarker in prostate cancer and could find use in several clinical settings including selection of patients for active surveillance and identification of patients at risk for recurrence after radical prostatectomy that would benefit from adjuvant radiation therapy [26]. AZGP1 expression provides independent, albeit modest improvement in predicting recurrence after radical prostatectomy, likely because of its association with adverse pathological features. However, this association with adverse pathology and the

strong association with outcome in Gleason score $\leq 3+3=6$ patients suggest its greatest utility could be selection of patients for active surveillance. One significant challenge will be developing clinical grade IHC assays for AZGP1 with well-characterized antibodies. Available antibodies against AZGP1 are polyclonal and ongoing availability for a clinical assay could be a significant issue. It is noteworthy, however, that loss of AZGP1 expression by IHC has been shown to be highly prognostic in several tumor types, including breast, gastric, and liver cancer [27–29]. Therefore, development of clinical grade assays with a well-characterized monoclonal antibody could find applications beyond prostate cancer.

The mechanisms by which loss of AZGP1 affects cancer aggressiveness are currently unknown. AZGP1 is an androgen-regulated gene, and AZGP1 protein is secreted at high levels in the prostatic fluid [30,31]. Gene-expression profiling suggests that androgens regulate pathways associated with terminal differentiation in the prostate including secretory proteins [32,33]. It is therefore possible that loss of AZGP1 merely reflects loss of terminal differentiation in more aggressive cancers. However, it is equally possible that AZGP1 plays an active role in suppressing carcinogenesis, particularly since it is prognostic across several tumor types. AZGP1 was originally described as a secreted member of the MHC1 family and it is possible it modulates immune response to the tumor [34,35]. Furthermore, in colon cancer cell lines, forced over-expression of AZGP1 results in down-regulation of the mTOR signaling pathway, decreased proliferation and invasion, increased apoptosis, and mitotic arrest [36]. In pancreatic cancer cell lines, AZGP1 has been shown to act as a tumor suppressor and loss of expression induces epithelial to mesenchymal transition, increases invasion in activates cell survival programs [37]. Clearly, additional work will be necessary to discover the role of AZGP1 in cancer progression.

Our study has some limitations. First, the relative age of some of the samples could affect RNA stability and influence RISH results, which might account for its lower predictive performance compared to IHC. Second, patient samples were collected retrospectively, and although we tried to limit biases by using a case control design, potential confounders are possible including changes in practice patterns or patient populations over time. Finally, rather than select cases that reflect the distribution of GS and RFS typical of the population of patients undergoing radical prostatectomy, we oversampled recurrent low grade (GS $3+3=6$), balanced recurrent and non-recurrent cases with GS $3+4=7$ and $4+3=7$ and oversampled non-recurrent GS ≥ 8 cancers. While this design has advantages in identifying biomarkers independent of GS, it will affect the weight of GS in univariable and multivariable models in predictions of clinical outcome.

CONCLUSIONS

Loss of expression of AZGP1 protein and RNA are associated with adverse pathological features at radical prostatectomy and are independently associated with RFS after surgery. Loss of expression is associated with OS and DSS in our cohort. Together these findings identify AZGP1 IHC as an independent prognostic marker in prostate cancer and provide the basis for development of a clinical grade assay. Further work will be necessary to define the relative performance of AZGP1 protein and RNA-based assays in assessing clinical outcome and to define the role of AZGP1 in suppressing cancer progression.

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RESEARCH ARTICLE

MUC1 Expression by Immunohistochemistry Is Associated with Adverse Pathologic Features in Prostate Cancer: A Multi-Institutional Study

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Abstract

Background

The uncertainties inherent in clinical measures of prostate cancer (CaP) aggressiveness endorse the investigation of clinically validated tissue biomarkers. MUC1 expression has been previously reported to independently predict aggressive localized prostate cancer. We used a large cohort to validate whether MUC1 protein levels measured by immunohistochemistry (IHC) predict aggressive cancer, recurrence and survival outcomes after radical prostatectomy independent of clinical and pathological parameters.

Material and Methods

MUC1 IHC was performed on a multi-institutional tissue microarray (TMA) resource including 1,326 men with a median follow-up of 5 years. Associations with clinical and pathological parameters were tested by the Chi-square test and the Wilcoxon rank sum test. Relationships with outcome were assessed with univariable and multivariable Cox proportional hazard models and the Log-rank test.

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Results

The presence of MUC1 expression was significantly associated with extracapsular extension and higher Gleason score, but not with seminal vesicle invasion, age, positive surgical margins or pre-operative serum PSA levels. In univariable analyses, positive MUC1 staining was significantly associated with a worse recurrence free survival (RFS) (HR: 1.24, CI 1.03–1.49, P = 0.02), although not with disease specific survival (DSS, P>0.5). On multivariable analyses, the presence of positive surgical margins, extracapsular extension, seminal vesicle invasion, as well as higher pre-operative PSA and increasing Gleason score were independently associated with RFS, while MUC1 expression was not. Positive MUC1 expression was not independently associated with disease specific survival (DSS), but was weakly associated with overall survival (OS).

Conclusion

In our large, rigorously designed validation cohort, MUC1 protein expression was associated with adverse pathological features, although it was not an independent predictor of outcome after radical prostatectomy.

Introduction

Prostate cancer (CaP) is the most frequently diagnosed cancer and the third leading cause of death from cancer among men worldwide [1]. Prostate specific antigen (PSA) testing has been used for screening and disease monitoring, such as in active surveillance or after therapy for CaP. However, for men with clinically localized CaP, PSA cannot reliably predict clinical outcomes, particularly since many men have a PSA level < 10 ng/ml at the time of diagnosis where PSA is not prognostic [2]. Therefore, additional biomarkers that are associated with clinical outcome are needed. The mucin family encompasses a diverse set of high molecular weight glycoproteins characterized by the presence of O-linked oligosaccharides to serine or threonine residues [3, 4]. MUC1 protein expression has been found to be significantly elevated in several cancers including CaP [4, 5] and is usually accompanied by altered glycosylation [6, 7]. In addition, MUC1 expression in cancer is usually characterized by a diffuse cytoplasmic staining pattern compared to apically restricted expression typically found in normal tissues [8–11]. MUC1 over-expression has been reported to allow malignant cells to evade host immunological defenses and to promote metastasis through a loss of cell–cell and cell–extracellular matrix contact [7, 12–16].

In CaP, MUC1 over-expression has been associated with increased risk of recurrence and adverse pathological findings in patients undergoing radical prostatectomy [5, 17–19]. We have developed a multi-institutional Tissue Microarray Resource of radical prostatectomy samples for definitive validation of biomarkers of prognosis that are independent of clinical and pathological features [20]. We have used this resource to validate several tissue-based candidate biomarkers of prognosis and evaluated whether their ability to prognosticate is independent of clinical and pathological features [21–26]. Our goal is to validate candidate biomarkers of prognosis to aid in the identification of patients with increased risk for tumor progression and poor survival outcomes after radical prostatectomy. Based on strong preliminary data implicating MUC1 expression as a marker of adverse outcome in CaP, we evaluated whether MUC1 expression by immunohistochemistry was associated with recurrence and survival after radical prostatectomy.

Materials and Methods

The study was conducted in accordance with IRB-approved protocols at each participating site (Stanford University, University of California San Francisco, University of Washington, University of British Colombia, University of Texas Health Sciences Center at San Antonio, Eastern Virginia Medical Center) and a materials transfer agreement for sharing of tissue microarrays, clinical information and tissue samples.

TMA cases and construction

The TMA cohort consisted of cases selected randomly by the study statistician (ZF) according to de-identified clinical data from each site such that recurrent and non-recurrent cases were balanced. Constraints were placed on case selection such that patients with recurrence and with Gleason score 3+3 = 6 and those with Gleason score 4+4 = 8 and no recurrence were oversampled. Details concerning case selection, tissue microarray construction and statistical considerations have been described elsewhere [20]. TMAs were constructed at each participating center using 1 mm cores and a standardized TMA layout. For each case, 3 cores of the highest grade cancer from the largest cancer area were used as well as one core of histologically normal prostate tissue from each case. In each TMA block at all sites, a common set of tissue cores (colon, tonsil, kidney, healthy prostate, and liver) was included as a staining control and for normalization across TMAs. Thereafter, the TMAs were baked and stored under nitrogen gas at each site.

Immunohistochemistry

Immunohistochemical staining was performed using freshly cut 5 micron sections from each site shipped to Stanford University and a commercial antibody for MUC1 (1:50 dilution; SC-7313, Santa Cruz Biotechnology) [20]. The digital image documentation of all stained slides was performed using the Leica SCN400 scanning system with the SL801 autoloader (Leica Microsystems; Concord, Ontario, Canada) at magnification equivalent to 40x. The images were transferred into the SlidePath digital imaging hub (DIH; Leica Microsystems). In parallel, separate TMA sections were stained with hematoxylin and eosin (H & E) and high molecular weight keratins (HMWK, 34bE12, Dako); these sections were scored for the presence of cancer in each core on the TMA as described previously [21–26]. A single pathologist (LF) scored MUC1 protein staining only in cores in which cancer was present as determined using the H & E and HMWK.

The immunohistochemical staining intensity for MUC1 was defined as absent, weak (faint cytoplasmic staining of scattered cells), moderate (intermediate or heterogeneous cytoplasmic staining in tumor cells), or strong (dense cytoplasmic staining of nearly all tumor cells) as shown in **Fig 1**.

Statistical methods

The clinical and pathological characteristics were comprised of age, pre-surgery PSA, post-surgical Gleason score, seminal vesicle invasion (SVI), extra-capsular invasion (ECE), and surgical margin status. Patient characteristics (e.g. race, lymph node status, etc.) with 25% or more missing were excluded from this analysis. Subjects with evaluable MUC1 staining, clinical and pathological data were included in the analysis.

The outcomes of interest included post-surgical recurrence-free survival (RFS), Disease-specific survival (DSS) and overall survival (OS). RFS was defined as the absence of PSA (biochemical) recurrence, local recurrence, CaP metastases, or death from CaP, with events determined at the earliest date noted after surgery. The endpoint of DSS was defined as death from

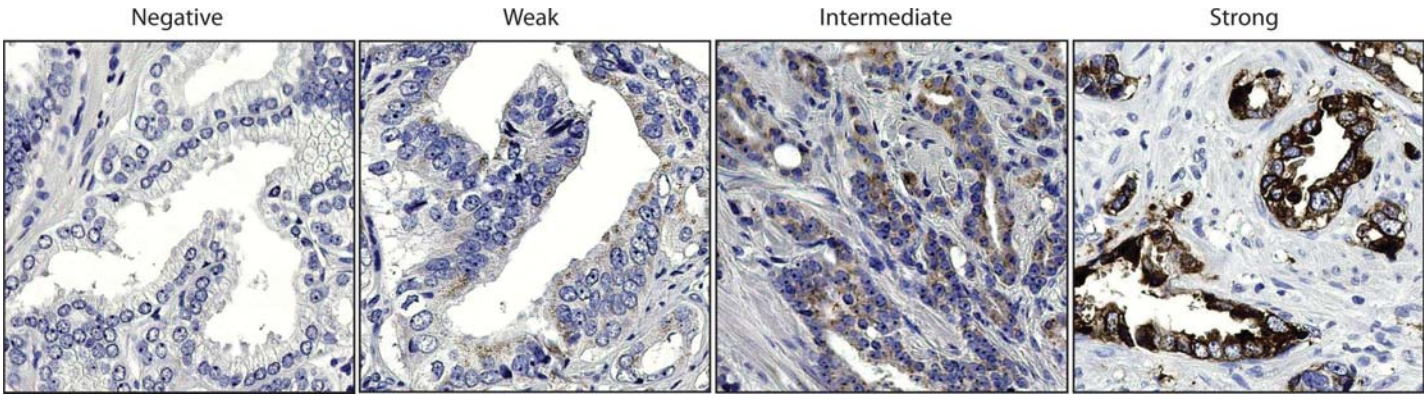

Fig 1. Immunohistochemical MUC1 staining in representative prostate cancer samples showing absent, weak, moderate and strong staining.

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CaP or development of metastatic disease. The endpoint of OS was defined as death from any cause. The date of surgery was considered as baseline for survival analysis. MUC1 IHC score was the maximum staining score of all cores for each patient. As described previously, MUC1 stained cases were divided into two groups, "negative" vs. "positive" (weak/moderate/strong staining), and compared to each other [5].

Descriptive statistics of patients' MUC1 protein expression by IHC were recorded as frequencies and percentages for the patient cohort. The association between MUC1 expression levels and categorical values was assessed by the Chi-square test. The Wilcoxon rank sum test was performed to evaluate the association between MUC1 expression and continuous variables. The Kaplan-Meier (KM) method was used to determine RFS, DSS and OS by MUC1 expression groups. We used the log-rank test to find significant differences between survival curves. Univariable and multivariable Cox regression analyses were performed to evaluate the prediction of MUC1 expression for each survival endpoint. Unweighted and weighted analyses were performed, with the latter accounting for the oversampling of patients with recurrence less than 5 years after surgery. All of the statistical tests were 2-sided, and the level of statistical significance was $P < 0.05$. Statistical analysis was performed using SAS version 9 (SAS Institute, Cary, NC). Kaplan Meier plots were created using Spotfire S+ 8.2 (TIBCO Inc., Palo Alto, CA). The complete dataset of clinical, pathological and staining data can be found in S1 File.

Results

The TMA was constructed from radical prostatectomy specimens from a total of 1,326 subjects. Of those cases, >25% of clinical or pathological data were missing in 51 cases (3.8%). MUC1 staining data were not available in 95 cases (7.2%) due to core loss or lack of cancer in the core samples. After excluding those cases, the remaining 1,180 cases with available clinical, pathological and IHC data constitute the cohort of the current study. Overall, 73.3% (865/1,180) showed absent MUC1 expression, 11.9% (140/1,180) showed weak expression, 9.2% (109/1,180) showed moderate expression, and 5.6% (66/1,180) showed strong expression. When MUC1 expression status was divided into "positive" and "negative" status, 26.7% of cases were scored with positive expression, whereas 73.3% of cases were negative.

MUC1 and clinicopathological features

MUC1 levels by IHC were tested for their association with clinical and pathologic features (**Table 1**). Initially we tested degree of staining (absent, weak, moderate, strong) for association

Table 1. MUC1 expression and clinical and pathological features.

	All	MUC1 Score		P-value
		Negative	Positive	
Population, n (%)	1180 (100%)	315 (26.7%)	865 (73.3%)	
Age at diagnosis, median (range), yr.	61 (35–80)	61 (35–78)	62 (42–80)	0.13*
Preoperative PSA level, mean (+/-SD), ng/mL	8.63+/-8.36	8.71+/-8.60	8.55+/-8.12	0.78*
Surgical margin status				0.19**
Positive, n (%)	347 (29.41)	259 (74.64)	88 (25.36)	
Negative, n (%)	666 (56.44)	471 (70.72)	195 (29.28)	
Unknown, n (%)	167 (14.15)	135 (80.84)	32 (19.16)	
Seminal vesicle invasion				0.57**
Yes, n (%)	78 (6.61)	55 (70.51)	23 (29.49)	
No, n (%)	1086 (92.03)	798 (73.48)	288 (26.52)	
Unknown, n (%)	16 (1.36)	12 (75.00)	4 (25.00)	
Extracapsular Extension				0.02**
Yes, n (%)	347 (29.41)	238 (68.59)	109 (31.41)	
No, n (%)	818 (69.32)	617 (75.43)	201 (24.57)	
Unknown, n (%)	15 (1.27)	10 (66.67)	5 (33.33)	
Gleason score				0.02**
< = 6, n (%)	494 (41.86)	382 (77.33)	112 (22.67)	
7a (3+4), n (%)	436 (36.95)	315 (72.25)	121 (27.75)	
7b (4+3), n (%)	135 (11.44)	93 (68.89)	42 (31.11)	
8–10, n (%)	107 (9.07)	69 (64.49)	38 (35.51)	
Unknown	8 (0.68)	6 (75.00)	2 (25.00)	

* Wilcoxon rank sum test

** Chi-square test

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with pre-operative clinical and pathological data and found no association of degree of staining and the presence of ECE, SVI, positive surgical margins, Gleason score and pre-operative PSA. However, patients showing a negative or weak status for MUC1 expression were younger than those with moderate or strong status. Since our goal was to validate whether MUC1 staining is a prognostic biomarker in CaP tissues, we simplified MUC1 staining into any positive staining (weak, moderate or strong) compared to absent staining since this was how MUC1 was scored in previous positive studies [5, 19, 27]. The presence of any MUC1 staining was associated with extracapsular extension (ECE) and higher Gleason score (GS) (**Table 1**). No significant association was observed between MUC1 expression and seminal vesicle invasion (SVI), patient age at the time of surgery, positive surgical margins (PSM) or pre-operative serum PSA levels. Lymph node status was missing for approximately half of the cases and therefore was not included in our analysis.

MUC1 and clinical outcomes after radical prostatectomy

Kaplan-Meier analysis showed that the strong MUC1 expression was significantly associated with worse RFS compared to negative, weak, or moderate MUC1 expression as shown in **Fig 2A** (P = 0.006, Log-rank test). When the cohort was stratified as either positive (weak, moderate, strong) or negative MUC1 staining, cases that were positive for MUC1 showed relatively slight but significantly worse RFS compared to those that were negative (**Fig 2B**). MUC1 expression was not associated with DSS when cases were grouped by their degree of staining

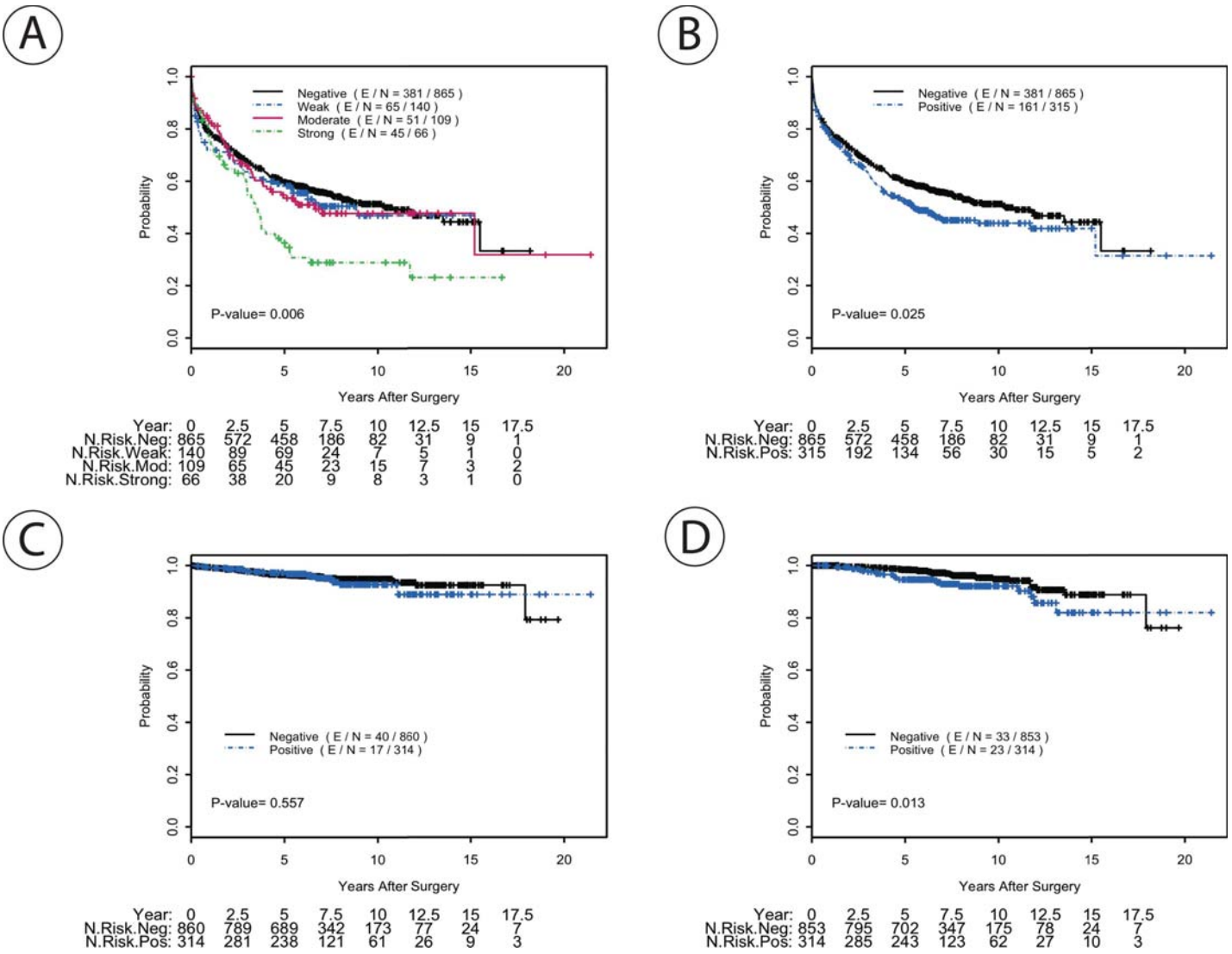

Fig 2. Kaplan-Meier plots of recurrence free survival (RFS) after radical prostatectomy **A)** for MUC1 staining gradient (absent, weak, moderate, and strong staining); **B)** for categorized MUC1 staining status (negative vs. positive); **C)** disease-specific survival for MUC1 positive and negative staining; **D)** Overall survival for the MUC1 positive and negative staining cases.

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(negative, weak, moderate, strong; not shown) or simply divided into positive or negative staining (Fig 2C). Patients with positive MUC1 staining had a slightly worse OS compared to those without (P = 0.013, Log rank test), although there was no significant difference in outcome when each staining group was considered individually (Not shown, P = 0.16, Log-rank test) (Fig 2D).

To further explore the relationship between MUC1 expression levels and clinical outcomes, we performed univariable Cox proportional hazards analysis for MUC1 expression (positive or negative), as well as clinical and pathological variables (Table 2). Patients with positive MUC1 staining had significantly a worse RFS (HR: 1.23, P = 0.02). RFS was also strongly associated with the presence of ECE, SVI, PSM, increasing pre-operative PSA and increasing GS, but not with patient age. DSS was associated with all of the clinical variables, but not with MUC1 staining status or patient age. OS was associated strongly with the presence of high GS,

Table 2. Univariate Cox proportional hazard model for recurrence-free survival, disease-specific survival and overall survival.

	Recurrence-free survival		Disease-specific survival		Overall survival	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Age	1.00 (0.99–1.01)	0.65	1.02 (0.99–1.06)	0.22	1.08 (1.03–1.12)	0.0004
Log(preoperative PSA)	2.17 (1.54–3.07)	<0.0001	2.17 (1.54–3.07)	<0.0001	1.68 (1.12–2.52)	0.01
MUC1						
Negative	Reference		Reference		Reference	
Positive	1.23 (1.03–1.49)	0.02	1.19 (0.67–2.08)	0.56	1.92 (1.14–3.33)	0.02
Surgical margin status						
Negative	Reference		Reference		Reference	
Positive	2.08 (1.74–2.48)	<0.0001	2.65 (1.43–4.91)	0.002	1.61 (0.95–2.72)	0.08
Seminal Vesicle Invasion						
No	Reference		Reference		Reference	
Yes	3.33 (2.63–4.35)	<0.0001	3.45 (1.82–6.67)	0.002	2.5 (1.18–5.26)	0.02
Extracapsular extension						
No	Reference		Reference		Reference	
Yes	1.92 (1.61–2.27)	<0.0001	1.96 (1.16–3.33)	0.01	1.69 (0.99–2.86)	0.05
Gleason score						
< = 6	Reference		Reference		Reference	
3+4 (7a)	1.43 (1.18–1.74)	0.0003	2.93 (1.43–6.00)	0.003	0.95 (0.48–1.88)	0.88
4+3 (7b)	2.39 (1.87–3.06)	<0.0001	3.71 (1.53–8.99)	0.004	1.42 (0.57–3.53)	0.45
8–10	2.39 (1.82–3.13)	<0.0001	7.30 (3.30–16.12)	<0.001	4.79 (2.52–9.11)	<0.0001

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age, and to a lesser extent with SVI, ECE, Pre-operative PSA and MUC1 staining (P = 0.02). Analysis of MUC1 expression degree by staining (negative, weak, moderate strong) slightly strengthened the association with RFS (P = 0.007), but did not change the association with DSS (P = 0.24) or OS (P = 0.06).

To evaluate whether MUC1 expression levels provided prognostic information independent of clinical variables, we performed multivariable Cox proportional hazards analysis (**Table 3**). MUC1 expression levels (positive/negative or absent/weak/moderate/strong) were not associated with RFS or DSS. As reported previously [21–26], RFS was associated with the presence of ECE, PSM, SVI, increasing Gleason score and higher pre-operative PSA. DSS in this cohort was only associated with Gleason score and pre-operative PSA levels. For OS, MUC1 did show a significant association (HR 1.82; 95% CI: 1.06–3.11; P = 0.03) as did GS and patient age. However, the associations between MUC1 staining and DSS and OS were limited by the relatively small number of CaP deaths or metastasis (n = 57) or deaths from all causes (n = 56).

Discussion

In a large multi-institutional clinical cohort, we have demonstrated that expression of MUC1 protein by immunohistochemistry is associated with extracapsular extension and high Gleason grade at the time of radical prostatectomy. This association confirms several smaller studies that have noted an association of MUC1 protein expression and increasing Gleason grade [8, 18, 28, 29], and disagrees with another study (N = 110) that showed no association of MUC1 expression with pathological features [30]. The association of MUC1 expression with adverse pathological features suggests that MUC1 could have utility as a biomarker for predicting tumor upgrading or upstaging. Because of sampling errors in biopsies, approximately 40% of Gleason score 3+3 = 6 cancers on pre-operative biopsy are found to be $\geq$ 7 at the time of

Table 3. The multivariate cox proportional hazard model for recurrence-free survival.

	Recurrence-free survival		Overall survival	
	HR (95% CI)	**P-value**	**HR (95% CI)**	**P-value**
Age			1.07 (1.02–1.11)	0.003
Log(preoperative PSA)	1.42 (1.22–1.67)	<0.0001		
MUC1				
Negative	Reference		Reference	
Positive	1.14 (0.92–1.42)	0.23	1.82 (1.06–3.11)	0.03
Surgical margin status				
Negative	Reference			
Positive	1.64 (1.32–2.03)	<0.0001		
Seminal Vesicle Invasion				
No	Reference			
Yes	2.10 (1.52–2.90)	<0.0001		
Extracapsular extension				
No	Reference			
Yes	1.30 (1.04–1.62)	0.02		
Gleason score		0.0001		0.0005
< = 6	Reference		Reference	
3+4 (7a)	1.20 (0.94–1.53)		0.89 (0.45–1.77)	
4+3 (7b)	1.92 (1.43–2.58)		1.17 (0.47–2.95)	
8–10	1.50 (1.07–2.09)		3.46 (1.76–6.78)	

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radical prostatectomy [31]. Under grading and under staging are significant challenges when selecting men with apparent low risk CaP for active surveillance, and likely account for significant rates of adverse reclassification for men while on surveillance [32]. The potential for MUC1 to predict adverse reclassification has been suggested by a demonstration that MUC1 expression independently predicted upstaging and upgrading in low risk prostate cancers incidentally discovered at the time of transurethral resection of the prostate. These cases were treated for benign prostatic hyperplasia and subsequently underwent radical prostatectomy [33].

Despite its association with adverse pathological features, MUC1 expression did not predict outcome independent of Gleason score, extracapsular extension, seminal vesicle invasion, positive surgical margins and pre-operative PSA levels. Previous reports have implicated MUC1 as a potential prognostic biomarker prostate cancer. Lapointe et al. showed that MUC1 expression was independently associated with RFS in a cohort of 225 patients after surgery, although in this study, Gleason score and stage were dichotomized as $\leq$3+4 compared to $\geq$4+3 and $\leq$pT2 vs. $\geq$pT3, respectively [5]. In a population-based study of 195 Swedish men managed by watchful waiting, MUC1 expression that deviated from normal was independently associated with disease specific survival [17]. However, deviation from normal was defined as staining above and below levels in normal prostate tissue, and cases with absent expression showed outcomes similar to those with high expression, a finding that differs from our findings and is difficult to explain biologically.

One significant challenge in developing MUC1 as a prognostic biomarker is that the protein is heavily glycosylated, and the glycoforms change in CaP compared to normal prostate tissue. In prostate cancer, as in many malignancies, MUC1 and other glycoproteins show truncated O-glycans and an increase in sialylation [18, 34]. The changes in glycosylation are driven in part by increased expression of the glycoprotein synthetic enzyme GCNT1 (β-1,6-N-acetylglucosaminyltransferase-1) in CaP compared to normal prostate tissues, which is associated with

an increase in sialylated MUC1 [6]. Using an antibody specific for sialylated MUC1, Arai et al. found high level expression by IHC was associated with higher grade and stage of prostate cancer as well as RFS and DSS [18]. However, the alterations in glycosylation patterns in cancer, as well as potential heterogeneity in the glycosylation patterns in cancer could complicate analyses of MUC1 expression in tissues and degrade its performance as a biomarker. For example, using a panel of antibodies specific to different glycoforms of MUC1, Burke et al. found significant differences in MUC1 expression and this dramatically affected the associations between MUC1 over-expression and pathological outcomes. Only the antibodies directed at less glycosylated forms of MUC1 demonstrated an association with adverse pathology [35]. The variation in staining results between the specific antibodies implies that there could be some heterogeneity in glycosylation patterns that could adversely affect the performance of MUC1 as a biomarker.

The finding of increased MUC1 expression in cancers with adverse pathologic features suggests that MUC1 could play a role in prostate cancer progression. MUC1 has been implicated in cancer progression in many model systems and has been shown to modulate cancer cell adhesion and migration, evasion of immune surveillance, and cancer cell signaling [34, 36]. In CaP, MUC1 expression is significantly higher in synchronous lymph node metastases compared to primary tumors and is correlated with adverse outcome [29, 37]. MUC1 expression has also been reported in prostate cancer metastatic to the bone [38]. Therefore, MUC1 might have an important role in prostate cancer progression, and has been considered as a potential therapeutic target in advanced disease [39].

Our study has some limitations. Patient samples were collected retrospectively and, although we tried to limit biases by using a case control design, potential confounders are possible including changes in practice patterns or patient populations over time. Rather than select cases that reflect the distribution of GS and RFS typical of the population of patients undergoing radical prostatectomy, we over-sampled recurrent low grade (GS 3+3 = 6), balanced recurrent and non-recurrent cases with GS 3+4 = 7 and 4+3 = 7 and oversampled non-recurrent GS$\geq$8 cancers. While this design has advantages in identifying biomarkers independent of GS, it will diminish the weight of GS in univariate and multivariate models in predictions of clinical outcome.

In summary, MUC1 expression is associated with extracapsular extension and higher Gleason score in men undergoing radical prostatectomy for clinically localized prostate cancer. However, MUC1 expression is not a prognostic biomarker since it is not an independent predictor of clinical outcome following surgery. Given its association with adverse pathology, MUC1 could have some role in selecting patients for definitive treatment who otherwise have features of low risk prostate cancer.

Supporting Information

S1 File. Raw clinical, pathological and staining data from the cohort.
(XLS)

Author Contributions

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Project administration: JB SH LN LF JS AHC DT PC MG DL PN IT LT ZF JM.

Supervision: JB SH LN LF JS AHC DT PC MG DL PN IT LT ZF JM.

Validation: JB WW HA LF PC JM.

Visualization: WW JB.

Writing – original draft: OE JB.

Writing – review & editing: JB WW HA LF PC JM.

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